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REVIEW

The dual role of fucosidases: tool or target

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Abstract

Regular intake of fucosylated oligosaccharides has been associated with several benefits for human health, particularly for new-borns. Since these biologically active molecules can be found naturally in human milk, research efforts have been focused on the alternative synthetic routes leading to their production. In particular, utilization of fucosidases to perform stereoselective transglycosylation reactions has been widely investigated. Other reasons that bring these enzymes to the spotlight are their role in viral infections and cancer proliferation. Since their involvement in the pathogenesis of these diseases have been widely described, fucosidases have become a target in newly developed therapies. Finally, activity disorders of biologically important fucosidases can lead to health problems such as fucosidosis. What is common for both mechanisms is the interaction between the enzyme and substrates in and around the active site. Therefore, this review will analyse different substrate structures that have been tested in terms of their interaction with fucosidases active sites, either in synthesis or inhibition reactions. The published results will be compared from this perspective.

Keywords Fucosidases · Enzymatic activity · Intermolecular interactions · Active site

Abbreviations

2'FL	2'-Fucosyllactose
3'FL	3'-Fucosyllactose
3FL	3-Fucosyllactose
Arg	Arginine
Asp	Aspartic acid
CAZy	Carbohydrate-Active enZymes
DNJ	1-Deoxynojirimycin
ES	Enzyme–substrate complex
Fuc	Fucose
FUCA1	Human lysosomal α-L-fucosidase
FUCA2	Human plasma α-L-fucosidase
FUC-*Be*	α-L-fucosidase from bovine epididymis
FUC-*Bk*	α-L-fucosidase from bovine kidney
FUC-*Bl*	Mutant α-L-fucosidase from *Bifidobacterium longum* spp *infantis*
FUC-*Cp*	α-L-fucosidase from *Clostridium perfringens* cloned and expressed in *Escherichia coli*
FUC-*Fg*	α-L-fucosidase from *Fusarium graminearum*
FUC-*Fo*	α-L-fucosidase from *Fusarium oxysporum* 377
FUC-*LrGG*	α-L-fucosidase from *Lactobacillus rhamnosus GG*
FucOS	Fucosylated oligosaccharides
FUC-*Psp*	α-L-fucosidase from *Pedobacter* sp. CAU209 cloned and expressed in *E. coli*
FUCs	α-L-fucosidases
FUC-*Tm*	α-L-fucosidase from *Thermotoga maritima*
GH	Glycosyl hydrolases
Glu	Glutamic acid
His	Histidine
HMOs	Human milk oligosaccharides
K_i	Enzymatic inhibition constant
K_m	Michaelis–Menten enzymatic constant
Nu	Nucleophile
PDB	Protein Data Bank
Phe	Phenylalanine
*p*NP	*para*-Nitrophenyl
*p*NPFuc	*para*-Nitrophenyl-α-L-fucopyranoside
Trp	Tryptophan
Tyr	Tyrosine
βFucF	β-L-fucosyl fluoride

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Introduction

Primary interest in α-L-fucosidases (FUCs) stems from the fact that fucose (Fuc) is present in several oligosaccharides and glycoproteins involved in several different biological processes, but also for their synthetic utility to access fucosylated oligosaccharides (FucOS) naturally present mainly in human milk. The latter has been found to confer crucial health benefits on the newly born. The most essential functions that these biomolecules are involved in include the enhanced absorption of minerals, development of the right gut microbiota, and prevention of microbial infections due to their antiadhesive properties, among others (Bode 2020; Cheng et al. 2021). When breastfeeding is not possible, the second-best option for infant growth is formula milk (Martin et al. 2016). Modern analytical methods have made it possible to establish the exact composition of human and bovine milk, specifically that bovine milk does not contain as much fucosylated oligosaccharides (FucOS) (Zeuner and Meyer 2020). This has led to improved infant products, marketed based upon increased FucOS content (Walsh et al. 2020). Such improvements were, in part, possible due to the development of synthetic pathways to produce biomolecules. Human oligosaccharides (HMOs) have been produced via transglycosylation reactions using glycosyl hydrolases as biocatalysts (Pérez-Escalante et al. 2022). However, the use of enzymatic routes to access a wider number of human oligosaccharides at the industrial scale has been hampered so far by low reaction yields (Zeuner et al. 2019).

The challenges associated with the usage of FUCs lies in the fact that their principal activity is hydrolysis of a glycosidic bond between the fucosyl residue and the non-reducing end of an oligosaccharide chain. The opposite reaction of the bond formation is thermodynamically disfavoured and requires such modification of the reaction conditions that the process could be forced towards the desired direction. Selection of an appropriate donor substrate and a nucleophile other than water are critical factors to accomplish the reaction (Faber 2011). The second limitation of the transglycosylation reaction catalysed by glycosyl hydrolases is that the product is also a substrate for the opposite reaction of hydrolysis which must be prevented by constant product removal (Zeuner et al. 2019). For synthetic approaches, fucosyl-derivatives with activated aglycon leaving groups have been tested, mainly *para*-nitrophenyl-α-L-fucopyranoside (*p*NPFuc) (Guzmán-Rodríguez et al. 2018; Shi et al. 2020).

In accordance with the Carbohydrate-Active enZYmes (CAZy) database, FUCs are glycosyl hydrolases (GH) that are grouped into four families, based on the amino acid sequence similarity between their catalytic domains: GH29, GH95, GH141, and GH151 (Drula et al. 2022). Among these enzymes, GH29 FUCs are better suit for FucOS synthesis as they maintain the configuration at the anomeric carbon that resembles those contained in the human body, in contrary to those from GH95 that invert the anomeric carbon configuration. According to phylogenetic studies, the GH29 family comprises enzymes expressed by a wide variety of organisms, particularly those from human origin, and are divided in subfamilies GH29A and B. Intra et al. (2007) evaluated 84 FUCs sequences from different organisms, reporting that there is a 30 to 50% similarity between the amino acid sequences of vertebrates and invertebrates. In turn, the FUCs sequence homology in mammals can vary from 50 to 94%. According to the structure presented by Sulzenbacher et al. (2004), PDB entry 1HL8, α-L-fucosidase from *Thermotoga maritima* (FUC-*Tm*) shows the highest homology with eukaryotic FUCs, specially with human FUCs, and it is considered the most representative model for higher organisms. More recently, Armstrong et al. (2022) found that when superimposed, nucleophilic and acid/base catalytic residues of human lysosomal α-L-fucosidase (FUCA1) and FUC-*Tm* occupy the exact same positions inside the active site. For these reasons, most enzyme-catalysed transfucosylation reactions have been tried with GH29 fucosidases and thus will be the main focus of the manuscript. Additionally, there is a higher level of classification of glycosyl hydrolases that groups the enzymes into clans based upon the way proteins fold, as protein folding is better conserved than amino acids sequence. In this classification, GH29 fucosidases present a $(\beta/\alpha)_8$ barrel folding that corresponds to clan GH-R, while those enzymes in the GH95 family present $(\alpha/\alpha)_6$ barrel that has no clan classification yet, which is also the case for GH141 and GH151 families.

In the most recent and comprehensive work of You et al. (2019) on the evolution of FUCs, 6208 different amino acid sequences were analysed using *Homo sapiens*, *Arabidopsis*, *Dictyostelium discoideum* and *Bacteroides thetaiotaomicron* as query sequences. The authors concluded that FUCs belonging to the GH29 family can be further divided into three subfamilies: (I) animals, including humans, as well as certain bacteria; (II) fungi and some bacteria; and finally, (III) plants and other bacteria. Noteworthy, the authors of both studies indicated the conserved amino acid sequence associated with the active site of the GH29 family contains a particular nucleophilic aspartic acid residue (Asp) involved in the catalytic reaction.

For these reasons, the focus of this review is to provide a reader with an overview of the chemistry of FUC activity in the interaction between the substrate/inhibitor structures and the active site of these enzymes. Firstly, the diversity of the most common substrate structures will be discussed. This

includes both donor and inhibitor molecules investigated in the synthesis of FucOS and in the inhibition of FUCs. Secondly, the active site of an enzyme model such as FUC-*Tm* and its closest vicinity will be assessed as part of an overall structural analysis. Finally, the interaction between the best substrates/inhibitors and the enzyme will be thoroughly discussed.

First part: fucosidases as tools and targets

Tools: donors for synthesis

The enzymatic synthesis of FucOS is an alternative approach to traditional chemical methods. It allows the formation of specific glycosidic bonds without the complex multistep routes required by protecting group chemistry. Oligosaccharides can be produced enzymatically either with fucosyltransferases or fucosylhydrolases. The former are characterized by high substrate specificity that allows precise synthesis of the desired oligosaccharide structures. Even though the use of fucosyltransferases have been steadily increasing over the years, their application is limited in in vitro experimentation due to the low availability and a need to use specific and expensive activated substrates. Transferases are used mostly in vivo and help construct different size oligosaccharides, while hydrolases can help to prepare building blocks for the construction of more complex and larger structures in vitro. Molecular biology advances are permitting the development of cell-factories that are becoming the most sought-after methodology for the preparation of smaller oligosaccharides (Faijes et al. 2019; Zeuner et al. 2019). In fact, according to Zeuner and Meyer (2020), the future prospect of fucosylated HMOs generation lies in the use of fermentation methodologies to produce foods, such as breast-milk replacements.

Optimization of the FUC-catalysed transglycosylation reactions involves selection of the adequate donor–acceptor pair. In this section of the manuscript the discussion will narrow towards donor structures since their recognition by the enzyme is a critical step for the successful transfer to the respective acceptor molecule. A good donor is defined as a molecule that carries a good leaving group attached to the anomeric carbon of the Fuc so that reaction times were short enough to prevent product hydrolysis. Depending on the enzymatic family, one donor would suit for either group, but not for the other. For example, enzymes of subfamily GH29B present a clear preference for donor substrates with α-1,3 and α-1,4 linkages. Furthermore, these enzymes seem to recognise a galactosyl residue in donor molecules, thus they do not act on *p*NPFuc (Fig. 1). Those from subfamily A present a rather relaxed regioselectivity for donors, and readily accept *p*NPFuc. This molecule is widely used in colorimetric assays to quantify hydrolytic activity of FUCs because once the glycosidic bond is broken, a yellow chromophore is produced (DiCioccio et al. 1982). Additionally, *p*NPFuc presents a structural feature that promotes its hydrolysis (the nitro group in *para*-position activates the glycosidic linkage) and, consequently, helps the transfer to a suitable acceptor (the energy liberated during the bond cleavage contributes to the reduction of the activation energy required). Along with its commercial availability, all these characteristics give this donor methodological advantages over natural substrates. Not to be overlooked, the main

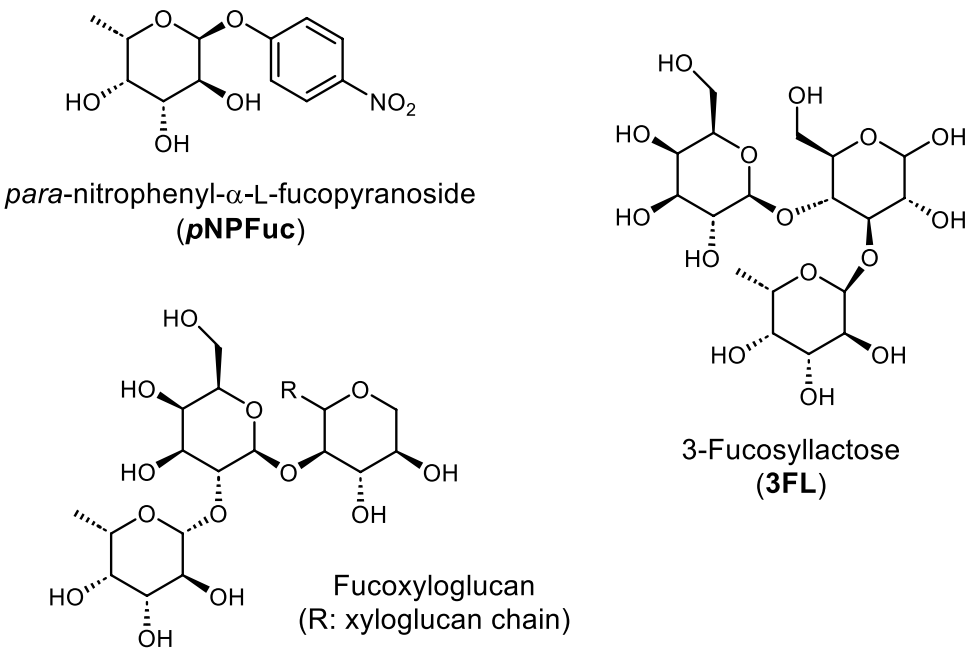


Fig. 1 Molecules used as fucosyl donors in enzyme catalysed transfucosylations

drawbacks when this molecule is used are two: the low solubility in aqueous conditions and the generation of undesired by-product, *para*-nitrophenol. Other donor structures that were investigated include monosaccharides activated with *orto*-nitrophenyl, halogens and methyl groups as substituents, as well as xyloglucans, and even with target transglycosylation products such as 3FL (Rodríguez-Díaz et al. 2013; Lezyk et al. 2016; Guzmán-Rodríguez et al. 2018; Zeuner et al. 2020).

There are several recent reviews that cover this topic in depth (Wan et al. 2020; Zeuner and Meyer 2020; Pérez-Escalante et al. 2022; Zheng et al. 2022), so in this section a general view on the subject will be provided to the reader, presenting a brief scope of the molecules used for this purpose. Some important works would be mentioned (Table 1). While the outcomes of the selected reactions presented in the Table are diverse, what was tried to emphasize were the donors that have been tried for FucOS preparation. In short, it is not the intention of this review to present a complete list of works on the matter but rather to introduce the reader to the field.

For example, Lezyk et al. (2016) expressed FUC encoding genes identified from soil metagenome in *E. coli*. Thus, the obtained enzymes were able to transfer Fuc from *p*NPFuc, although with rather low overall yields (Entry 1). Interestingly, these enzymes also catalysed the autocondensation of fucosyl moieties while hydrolysing the donor. Saumonneau et al. (2016) used 3FL as donor to transfucosylate three different acceptors, including 2'FL, with mutant enzymes from *Bifidobacterium longum* ssp. *infantis* (FUC-*Bl*, entry 2, PDB 3UES), which belongs to GH29B family. The yields ranged between 17–21%. It is noteworthy that equimolar amounts of donor and substrates were used. Normally, an excess of acceptor molecules helps to force the equilibrium towards synthesis.

One of the highest yields reported for 2'FL production was obtained using *p*NPFuc to transfucosylate Lac with FUC-*Tm* (Guzmán-Rodríguez et al. 2018; Entry 3). The authors were able to achieve 25% yield when the ratio acceptor:donor was higher than 150. The same research group tested a FUC obtained from *Lactobacillus rhamnosus* GG (FUC-*LrGG*) using the identical donor and galactose, lactulose, and Lac as acceptors, that resulted in 0, 21, and 25% product yields, respectively (Entry 4). Although the authors could not confirm that the transfucosylation product to Lac was 2'FL or a different fucosylated molecule, they determined that the obtained compound corresponded to a trisaccharide. On the other hand, Zeuner et al. (2018) expressed seven enzymes from different sources to test their transfucosylation activity using different donors. It was shown that α-L-fucosidase from *Clostridium perfringens* (FUC-*Cp*) was capable of transferring Fuc from 3FL to form a pentasaccharide at 37% yield (Entry 5). Authors achieved the best conversion yield when the amount of acceptor was 10-times higher than that of donor. Nevertheless, using equimolar amounts of both substrates led to yields close to 30%.

In another study, α-L-fucosidase from *Fusarium graminearum* (FUC-*Fg*) was demonstrated to accept xyloglucan from citrus as a donor for 2'FL production (14%), which was a promising result obtained for this renewable donor. Further studies with the enzyme, which was mutated within specific positions taken from other GH29 FUCs (FUC-*Bl* and FUC-*Tm*), showed that the structural change increased the regioselectivity, although it did not outperform the wild-type FUC-*Fg* in transfucosylation activity. Authors also found that previous depolymerization of the xyloglucan donor increased the overall yields in the enzymatic reactions.

Table 1 Results of prominent transfucosylation reports

ENTRY	DONORS	ENZYME[a]	YIELD (%)[b]	REFERENCE
1	*p*NPFuc	FUC-*Tm*	0.6–6.4	Lezyk et al. 2016
2	3FL[c]	Mutant FUC-*Bl*	17-21[d]	Saumonneau et al. 2016
3	*p*NPFuc	FUC-*Tm*	25.2	Guzmán-Rodríguez et al. 2018
4	*p*NPFuc	FUC-*LrGG*	21–25	Escamilla-Lozano et al. 2019
5	3FL and xyloglucan	FUC-*Cp* and *Fg*	37 and 14[d]	Zeuner et al. 2018
6	Xyloglucan	FUC-*Fg*	24	Zeuner et al. 2020
7	*p*NPFuc	FUC-*Psp*	14.5 (2'FL), 70.5 (3'FL)[e]	Shi et al. 2020

[a]Enzymes: FUC-*Tm* (α-L-fucosidase from *Thermotoga maritima*), FUC-*Bl* (mutant α-L-fucosidase from *Bifidobacterium longum* spp. *infantis*), FUC-*LrGG* (α-L-fucosidase from *Lactobacillus rhamnosus GG*), FUC-*Fg* (α-L-fucosidase from *Fusarium graminearum*), FUC-*Cp* (α-L-fucosidase from *Clostridium perfringens* cloned and expressed in *Escherichia coli*) and, FUC-*Psp* (α-L-fucosidase from *Pedobacter* sp. CAU209 cloned and expressed in *E. coli*). [b]Yields are for different products: entries 1, 3, 4, and 7 (2'-fucosyllactose, 2'FL), entry 2 (different FucOS), entry 5 (fucosylpentasaccharide and 2´FL), entry 6 (mixture of 2'FL and a fucosyltrisaccharide). [c]3FL stands for 3-fucosyllactose. [d]Used different acceptors than lactose. [e]Authors found a 2'FL isomer, 3'-fucosyllactose (3'FL)

FUC-*Fg* allowed for 18% conversion of 2'FL, while mutant variants showed the product content ranging from 6.7 to 23% (Zeuner et al. 2020; Entry 6). Noteworthily, there was also an unidentified trisaccharide regioisomer produced in all cases, which would increase the yields from 18 to 24% (FUC-*Fg*) and from 23 to 37% (most productive mutant variant). Finally, Shi et al (2020) synthesized both 2'FL and 3'FL with a recombinant FUC from *Pedobacter* sp. CAU209 (FUC-*Psp*) that was cloned and expressed in *E. coli* (Entry 7). Surprisingly, this enzyme was able to use *p*NPFuc for the synthesis of two fucosylated trisaccharides, 2'FL and 3'FL, with the remarkably higher yield for the latter, being 14.5 and 70.5%, respectively. Thus, this work presents the highest conversion yields obtained so far among wild-type FUCs in transfucosylation reactions.

While the strategy to improve the outcome of transfucosylation reactions through genetically modified enzymes has proven to be of interest (Wada et al. 2008), enzymatic hydrolysis of products affects the overall efficiency of the transfucosylation reactions. One way to overcome this problem involves single mutations of catalytic residues in FUCs to prevent the hydrolysis, although the enzymatic activity could be negatively affected. This approach is known as glycosynthase technology and has been applied to biotransformations with retaining endo/exo-β-glycosidases and exo-α-glycosidases. Mutated enzymes (fucosynthases) require the usage of activated fucosyl donors with the opposite anomeric placement to catalyse the transfer to the acceptor molecule, such as β-L-fucosyl fluoride (βFucF). These types of derivatives have been thoroughly studied. Numerous authors have reported the application of glycosyl fluoride molecules with the opposite anomeric configuration to mimic formation of the glycosyl-enzyme intermediate; transglycosylation activity in the active site resulted in the accumulation of the product, which the enzyme was uncapable to hydrolyse (Malet and Planas 1998; Williams and Withers 2000; Okuyama et al. 2002; Wada et al. 2008; Luijkx et al. 2021). Sakurama et al. (2012) reported the synthesis of FucOS with yields ranging between 6–41% when using βFucF as donor and lactose, 2'FL and lacto-*N*-tetraose as acceptors with a 4:10 [donor:acceptor] ratio. The major drawbacks of this methodology included fluoride liberation and the spontaneous hydrolysis of the donor.

Targets: inhibitors of enzymatic activity

Impaired activity of α-L-fucosidases

As described previously, fucosylated oligosaccharides are linked to several benefits including positive effects on the health of new-borns, well-documented prebiotic activity, as anti-adhesive agents of intestinal epithelial cell wall, stimulation of brain development, and as immune modulators (van Leeuwen 2019). They have also been associated with a reduced incidence of asthma, allergies, inflammatory bowel disease, type 1 diabetes, celiac disease, and leukaemia (Wicinski et al. 2020).

Conversely, there are various disorders stemming from changes in the metabolic activity of FUCs; alterations in fucosylation have been observed during pregnancy or in some pathological processes. Accumulation of Fuc-containing glycoconjugates, due to the absence or deficiency of FUCs, induces the recognition of α-L-Fuc moieties by specific lectins. This leads to a neurovisceral condition known as fucosidosis, which is a rare lysosomal storage disorder inherited in an autosomal recessive pattern (Michalski and Klein 1999; Saleh-Gohari et al. 2018). Recent works suggest that changes in the plasma level of FUCA1 can be associated with Sjögren's syndrome, an autoimmune, chronic, and systemic disorder characterized by lymphocytic infiltration of the exocrine glands and a remarkable B-cell hyperactivity (Mavragani and Moutsopoulos 2014; Endreffy et al. 2019). The presence of certain FUC, such as human plasma α-L-fucosidase (FUCA2), was found to be necessary for adhesion of *Helicobacter pylori*, particularly to gastric cancer and duodenal ulcer specific strains (Liu et al. 2009; Miura et al. 2019). It has been also suggested that these enzymes could play a role in SARS-CoV-2 infection (Liang et al. 2021).

Moreover, Fuc and its derivatives play a key role in biological processes not necessarily related to diseases. For example, these molecules have been shown to inhibit sperm–egg interactions in humans. Therefore, specific FUC inhibitors are expected to be powerful tools in elucidating the biological role of this enzyme in spermatogenesis and sperm maturation (Venditti and Bean 2009).

Inhibitory molecules have been extensively proposed as a therapeutic strategy to control the problems associated with malfunctioning/dysfunctional enzymes and/or stop their activities (Copeland et al. 2007). This has triggered the search for the best FUC inhibitors, as they have vital clinical applications acting as medical agents.

α-L-Fucosidase inhibitors

FUC inhibitors have gained a remarkable importance as medicinal therapeutics owing to the diversity of the biological processes these enzymes take part in, as previously mentioned. Among the molecules tested to date, the most studied has been 1-deoxynojirimycin (DNJ, Fig. 2). DNJ presents characteristic structural features formed when the enzyme–substrate complex is produced that resemble those of the oxocarbenium ion, mainly the nitrogen atom inside the ring and its ability to support a positive charge. This cation can interact through electrostatic forces with catalytic residues Asp and glutamic acid (Glu) (Ikeda and Takahashi 2007).

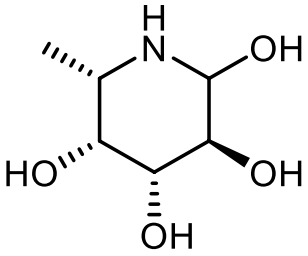


Fig. 2 Structure of 1-deoxynojirimycin (DNJ)

In fact, since FUCs are highly stereospecific at the anomeric centre, their anomer-selective inhibitors should mimic the oxocarbenium cation, a key intermediate where the anomeric configuration is still present, preceding the glycosidic bond cleavage (Ikeda and Takahashi 2007). For that purpose, several different structures have been tested for enzymatic inhibition, including, among others, aminocyclopentitols, iminocyclitols (pyrrolidines, pyrrolidines, azepanes), azafagomines, and carbasugars (Fig. 3). Relevant examples of each group will be briefly addressed in the following section. Currently, to our knowledge, there are no specific clinical studies on the use of these particular molecules, although they have been extensively studied in vitro. According to Moreno-Clavijo et al. (2011), FUCs in vitro inhibitors function as probes to test their potential activity and, thus, they can be used to develop potential therapeutic agents. Deeper knowledge of both molecular structure and enzymatic activity could lead to the development of suitable selective inhibitors.

Aminocyclopentitols

Aminocyclopentitols are polyhydroxylated cyclic amines that are effective anomer-selective fucosidase inhibitors, as they act as mimics of α- or β-configured protonated glycosides. The aminocyclopentitol scaffold/moiety is commonly found in many natural bioactive molecules such as mannostatin A, trehazolin, allosamidin, and carbocyclic nucleosides (Boss et al. 2000; Das and Panda 2013). As aminocyclopentitols have shown to be good β-galactosidases and β-glucosidases inhibitors, Blaser and Reymond (2001)

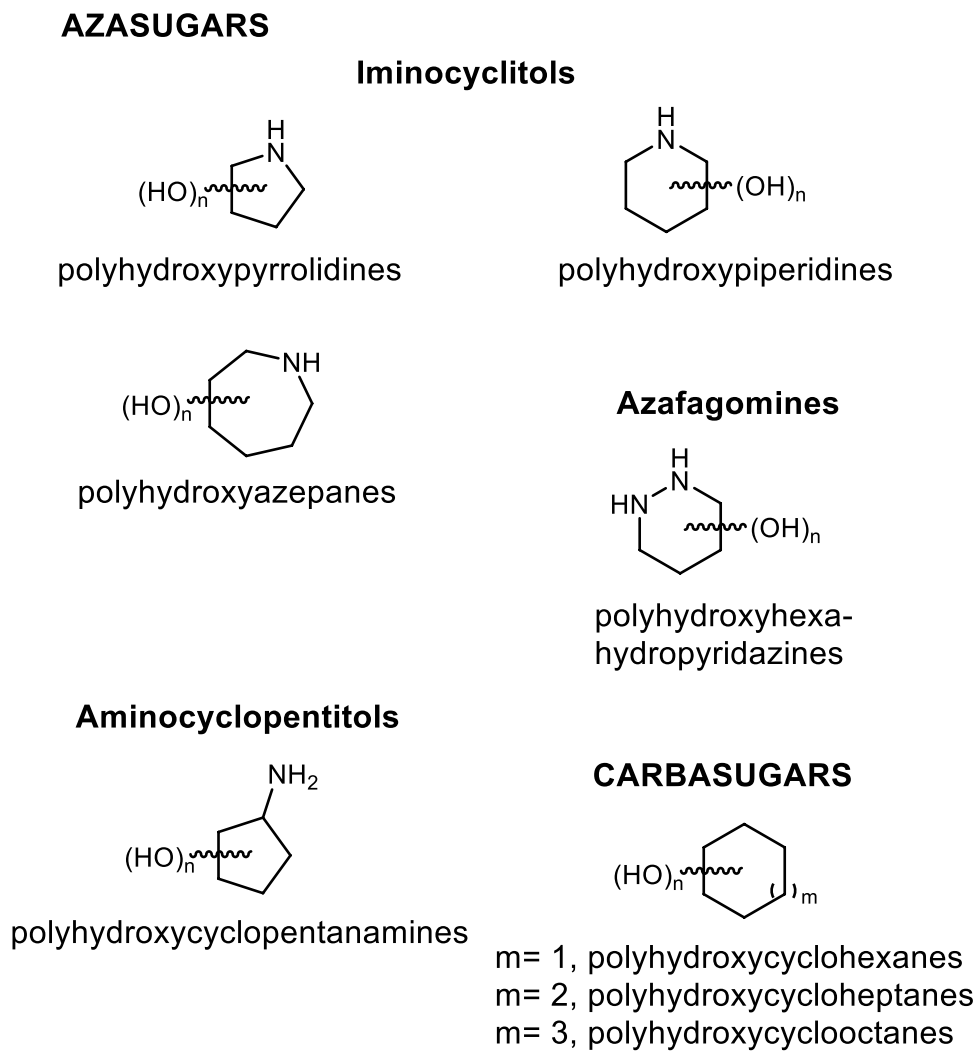
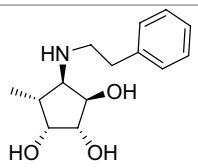
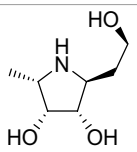
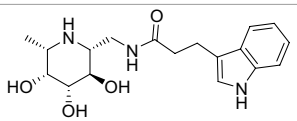
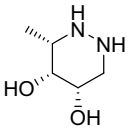
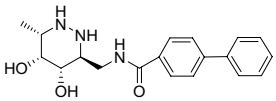
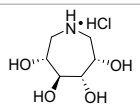
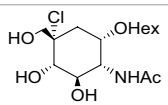


Fig. 3 General structures of FUCs inhibitors (n = 2 to 4)

Table 2 Several remarkable structures of the most common inhibition moieties used of FUCs

ENTRY	STRUCTURE	K_i (pM)	Reference
1 Aminocyclopentitols		8 x 10^4 FUCA1[a]	Blaser and Reymond 2001
2 Polyhydroxylated pyrrolidines		8 x 10^3 FUC-*Bk*[b]	Chevrier et al. 2006
3 Polyhydroxylated piperidines		0.47 FUC-*Tm* (time dependent)	Wu et al. 2010
4A Polyhroxylated hexahydropyridazines (azafagomines)		6.3 x 10^5 FUCA1[a]	Jensen et al. 2002
4B (azafagomines)		1 x 10^6 FUC-*Bk*[b]	Moreno-Clavijo et al. 2010
5 Polyhydroxylated azepanes		4.1 x 10^4 FUC-*Bk*[b]	Li et al. 2008
6 Carbasugars		*c*	Narayana et al. 2018

[a]FUCA1: FUC-Human lysosomal; [b]FUC-*Bk*: FUC-Bovine kidney; [c]IC_{50} value for FUC-*Bk* = 0.42 mM

synthetized different aminocyclopentitols with an α-L-fucosyl core (Table 2, entry 1) as these compounds incorporate an additional stereogenic unit at NH_2-substituted C-atom producing the α- or β-anomeric configuration at the glycosidic bond. This cyclic structure showed competitive inhibition and the best K_i values for all four FUCs tested (bovine kidney, FUC-*Bk*; bovine epididymis, FUC-*Be*; *Fucosarium oxysporum* 377, FUC-*Fo*, and FUCA1). FUCA1 was best inhibited (9×10^4 pM) while the others were consistently presenting values 10 times higher than this. The authors suggested that this molecule occupies the active site as an analogue of the transition state, with the phenylethylene group interacting with aminoacids residues that normally interact with the leaving group.

Iminocyclitols

Iminocyclitols (also called iminosugars or azasugars) are carbohydrate analogues that mostly carry a nitrogen atom at the position of the endocyclic oxygen. These structures have been found widely distributed in nature and are normally classified based on their ring size. They also represent an important glycosidase inhibitor class due to their attractive glycomimetic activity. Due to their protonated form at physiological pH that mimics the "oxocarbenium-ion-like" transition state, they usually offer high affinity toward glycosidases (Nishimura 2009; Wu et al. 2010; Moreno-Clavijo et al. 2013). This class of compounds comprises polyhydroxypyrrolidines, piperidines and azepanes, and some examples of each group are presented in the next lines:

Polyhydroxypyrrolidines

The five-membered aza-rings called pyrrolidines have the furanose-ring oxygen replaced by a nitrogen atom, acting as sugar mimic. This structural change has an important effect on their biological properties since these molecules bind better to glycosidases when compared to their parent carbohydrate substrates. They have been tested as potent inhibitory agents against FUCs (Kotland et al. 2011). One of the most

potent molecules of this type known so far was prepared by Chevrier et al. (2004) and its structure is shown in Table 2 (entry 2). Of the several pyrrole-like structures tested, which all showed competitive inhibition, this molecule which has a hydroxymethylene branch attached to the cycle, displays the lowest values of inhibition (8×10^{-3} pM). The authors suggested this could be due to a hydrophilic interaction in the active site, although it does not seem to be what is occurring with other inhibitors.

Polyhydroxypiperidines

Six-membered azasugars analogues possess hydroxyl groups with configurations like those of natural sugars. The first known structure of this type was nojirimycin, a labile molecule that can suffer an elimination reaction thus losing its biological activity. DNJ, was later isolated from the *Moracae* tree and proved to be more stable. Since then, DNJ was used as a starting model to prepare new inhibitors. This group represents most of the best-known glycosidase inhibitors in general and FUCs in particular (Ramesh 2020). Wu et al. (2010) reported ten inhibitor complexes tested on FUC-*Tm* (as it is the closest bacterial enzyme relative to mammalian α-L-fucosidase). From all these structures, the authors found the most potent FUCs inhibitor known to date (Table 2, entry 3), which presents inhibition constant values (K_i) in the sub picomolar range after the enzyme is allowed to stabilize. Thus, this activity is said to be "time-dependent". More on the topic later in the manuscript.

Polyhydroxyazepanes

Although most of the best FUCs inhibitors known are five or six-membered cyclic imines, there is another group that presents a larger structure. Tetrahidroxylatedazepanes are seven-membered *N*-heterocycles, that have been known for a good part of the last century to be glycosidase inhibitors. These structures present more ring flexibility and this could potentially lead to easier accommodation in the active site of FUCs (Li et al. 2009). Aside from enhanced ring flexibility when compared to 5 and 6-membered cycles, such inhibitory activity may also be related to several other distinct characteristics of tetrahydroxylatedazepane structure: the methyl groups located in the ring or the additional functionalization opportunities due to the extra in-the-ring carbon allowing supplementary binding interaction (Shih et al. 2011; Taghzouti et al. 2015).

One good example of a polyhydroxylatedazepane is presented in Table 2 (entry 5). Li et al. (2008) prepared several different polyhydroxylated azepanes and found that FUC from bovine epididymis was inhibited through a competitive inhibition with a K_i of 4.1 nM (Li et al. 2008). This azepane structure was not expected to present such an activity since it lacks both a methyl group and L-Fuc configuration.

Azafagomines

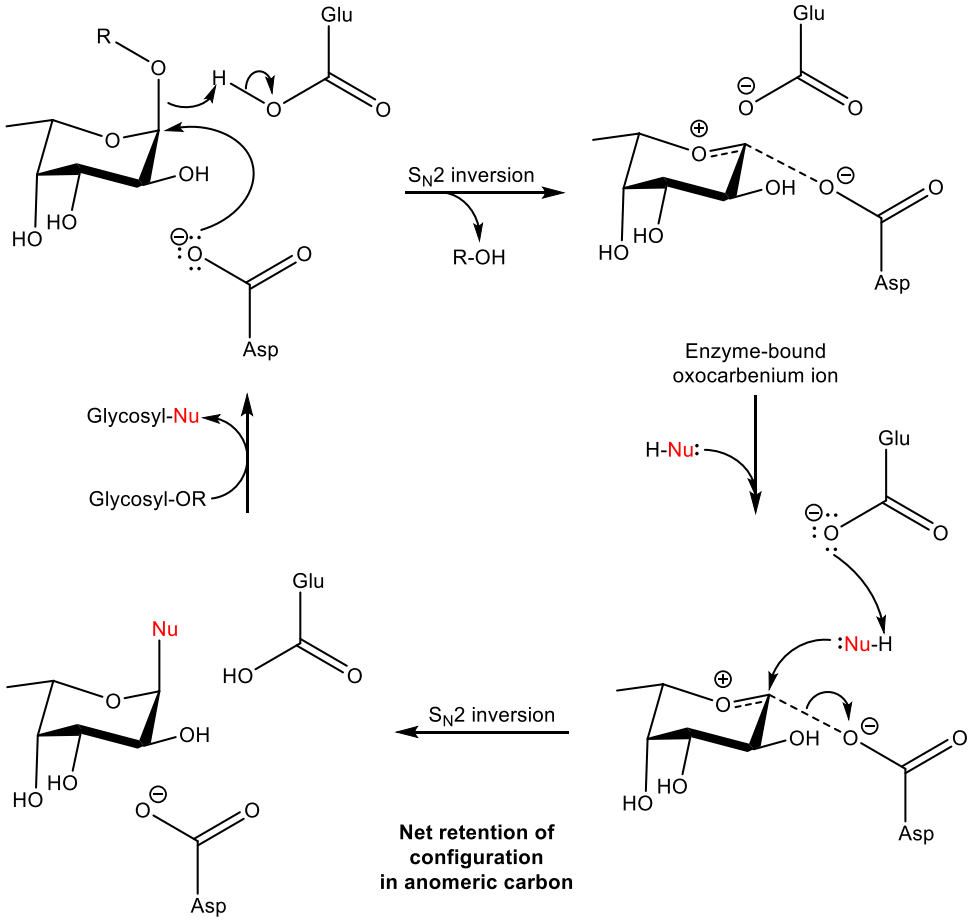
Azafagomines are six-membered monosaccharide analogues where C-1 and C-2 atoms have been replaced by a hydrazine moiety and present a hybrid structure between an azasugars and an iminosugar (Bols et al. 2007). Jensen et al. (2002) synthesized several azafagomines and assessed their activity towards different glycosidases such as FUCA1 and FUC-*Bk*. The structure that presented the best inhibitory results is shown in Table 2 (entry 4A). The values are still higher than those of some piperidines and the authors thought at the time that this could be a consequence of the missing hydroxyl group next to the nitrogen atom. Nevertheless, some years later Moreno-Clavijo et al. (2010) tested a molecule with a hydroxymethylene group bound to C-3 and found K_i values one order of magnitude lower for inhibition of FUC-*Bk* (Table 2, entry 4B).

Carbasugars

Carbasugars are carbocyclic analogues of carbohydrates, in which a methylene (-CH_2-) unit substitutes the cyclic oxygen atom of the sugar core. Carbasugars act as mimic of natural sugars; they lack the acetal linkage that is transformed into a non-hydrolysable ether. These compounds are very stable and do not react in any typical carbohydrate reaction, such as mutarotation, thus they were thought to represent remarkably interesting candidates as competitive inhibitors (Sollogoub and Sinaÿ 2005; Wadood et al. 2018). Despite being mostly synthetically prepared, these molecules are scarcely produced by some microorganisms (Wadood et al. 2018). Due to the structural characteristics of these molecules, carbasugars are readily recognized by glycosidases but do not interact strongly enough to be considered effective inhibitors. For this purpose, some modifications to their structure should be made. For example, creating insaturations on the ring and/or attaching amine groups. These alterations have already been tried with some antiviral pharmaceuticals such as oseltamivir (Narayana et al. 2018). For FUC inhibition by carbasugars there are some works on the subject, although inhibition values are rather high. Narayana et al. (2018) prepared *N*-acetylglucosamine carbasugar analogues of several different glycosidases. One of them, entry 6 (Table 2), presented some inhibitory activity for FUC bovine kidney but in the mM range, although this structure could very well be classified as an aminocyclitol.

Second part: how do fucosidases work?

FUCs are classified as hydrolases, in particular exoglycosidases that catalyse hydrolysis of α-L-Fuc from the non-reducing end of FucOS and fucoglycoconjugates. As already mentioned, FUCs belong to four families: GH29,



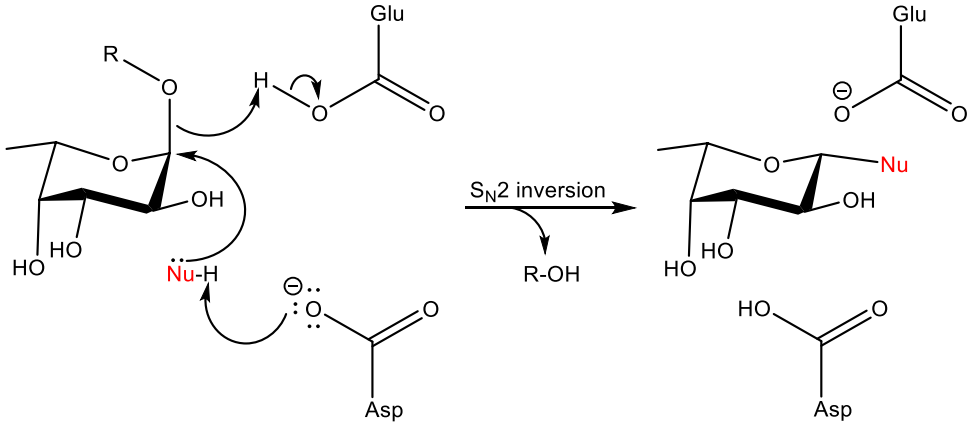
Scheme 1. Catalytic mechanism of the α-L-fucosidases from the GH29 family. R = carbohydrate, *p*NP, other; Nu = nucleophile molecule (water, carbohydrate)

GH95, GH141 and GH151 (Drula et al. 2022). The GH29 family comprises FUCs that hydrolyse linkages like [α-(1,2)] between Fuc and galactose; [α-(1,3)] to glucose; α-(1,3), α-(1,4), α-(1,6) linkages to *N*-acetylglucosamine residues, and α-(1,3), α-(1,4) between Fuc-units in fucoidan. These enzymes follow the mechanism of retaining the anomeric configuration in the double displacement reaction of the *O*-fucosyl bond. Two amino acid residues play a key role in the reaction: Glu acting as Lewis-acid/base and Asp acting as a nucleophile (Scheme 1). The first step consists of the O atom activation in the leaving group by Glu that facilitates the further nucleophilic attack to the anomeric carbon by Asp and formation of the glycosyl-enzyme intermediate. Then this structure undergoes a second nucleophilic attack, either by a water molecule resulting in a hydrolysis reaction or by another acceptor resulting in the formation of fucosylated oligosaccharide (transglycosylation reaction), which is liberated to the reaction medium (Sulzenbacher et al. 2004; Zeuner et al. 2014).

The GH29 FUC family comprises two subfamilies (A and B) according to the substrate specificity and phylogenetic properties. Enzymes that belong to subfamily A show relatively low substrate specificity, while those from subfamily B are highly regiospecific towards α-(1,3) or α-(1,4) linkages when galactose is branched next to the fucosylated saccharide (Shaikh et al. 2013; Saumonneau et al. 2016; Zeuner et al. 2018).

As for the GH95 family, its members follow a single-displacement mechanism via S_N2-type reaction. As shown in Scheme 2, the aglycone moiety (ROH) is formed due to a nucleophilic attack of an enzyme-activated molecule (NuH) (Faber 2011; Koval'ová et al. 2019) Similar to the GH29 FUCs, two amino acid residues in the active site participate in the catalysis: the carboxyl groups of Glu and Asp acting as Lewis acid and base, respectively. Due to the differences in the reaction mechanisms, and the specificities involved in molecular interactions, only GH29 FUCs are suitable biocatalysts for the synthesis of fucosylated oligosaccharides (Koval'ová et al. 2019; Saumonneau et al. 2016).

There are few available reports on the reaction mechanisms of the remaining GH141 and GH151 FUC families (Koval'ová et al. 2022). For example, FUC BT1002 from *Bacteroides thetaiotaomicron*, classified into the GH141 group, was proposed to catalyse a double displacement reaction where two aspartates, namely Asp523 and Asp564 acted as nucleophile and general acid/base, respectively (Ndeh et al. 2017). On the other hand, the *Paenibacillus thiaminolyticus* enzyme that belongs to the GH151 family has been



Scheme 2. Catalytic mechanism of the α-L-fucosidase from the GH95 family. R = carbohydrate, *p*NP, other; Nu = nucleophile molecule (water, carbohydrate)

reported to both hydrolyse and transfucosylate. Thus, it was inferred that this catalyst presents a retaining mechanism like that of GH29 FUCs (Benešová et al. 2013; Koval'ová et al. 2022).

Third part: What is with the neighbour? Analysis of the interaction between FUC-*Tm* and substrates/inhibitors

The study of structural models of enzymes, specifically their active sites, has been increasing dramatically in recent years. This stands out after a simple search in the CAZy database where information related to enzymes involved in various catalytic processes with carbohydrates is found. Additionally, in the Protein Data Bank (PDB), one can find the structural models of the most studied enzymes both in their apo form, or free form, and forming complexes with different compounds, usually inhibitors.

According to the data on the CAZy webpage, up to October 2022, the GenBank database has 9507 amino acid sequences registered belonging to GH29 FUCs. Additionally, the PDB has 69 defined structural models of 15 different enzymes (Drula et al. 2022). From the sequences and models, the active site region of the GH29 family of FUCs has been studied and characterized; there is a conservation of the amino acids that forms the active site. This is particularly useful when evaluating the way substrates bind or inhibitors interact with the catalytic activity of these enzymes (Shaikh et al. 2013; Koval'ová et al. 2019; You et al. 2019; Grootaert et al. 2020). For example, Koval'ová et al. (2019) reported that the active site of a FUC isoenzyme 1 from *Paenibacillus thiaminolyticus* is made up of 15 residues of which 7 are aromatic in nature, such as tryptophan (Trp), phenylalanine (Phe) and tyrosine (Tyr). There are also 3 histidines (His) and one arginine (Arg), which are basic residues that strengthen the interaction between the enzyme and the substrate. Of all these residues, four, one His and one Trp, as well as the catalytic residues, Asp and Glu, are preserved in the same positions in all FUCs of the GH29 family. It is noteworthy that the structural models of FUCs show that these residues are in the same coordinates but in distinct positions according to the amino acid sequence. Noteworthily, Armstrong et al. (2022) have just recently identified the amino acid residues vital for catalysis on FUCA1 and established that, contrary to most of the GH29 FUCs, this enzyme uses an Asp residue as the proton donor instead of a Glu. Despite these differences, when the structures of this enzyme and that of FUC-*Tm* are superimposed, the positions the catalytic residues occupy inside the active site are almost identical.

Based on the above, several authors have taken as a reference the FUC-*Tm* model, since, of the 66 structural models reported in the CAZy, 14 are of this enzyme. In addition, as already mentioned, it is the most representative FUC because it presents a 38% homology, particularly with the region of the active site, with human FUC, which also belongs to the GH29 family (You et al. 2019; Chkioua et al. 2021). In this way, the structural model of the FUC-*Tm* facilitates the study of the possible interactions of the human FUC. This includes the factors that influence the disease of fucosidosis, caused by a deficiency of the FUC, as well as other conditions caused by the activity of this enzyme (Shaikh et al. 2013; Grootaert et al. 2020; Chkioua et al. 2021).

The first structural models of FUC-*Tm* were reported by Sulzenbacher et al. (2004), where the authors crystallized the enzyme in its apo form (PDB: 1HL8) and forming complexes with both Fuc (PDB: 1ODU) and fucosyl fluoride (PDB 1HL9). From these structural models and with the use of computational tools the authors were able to locate the region of the active site, which is in the *N*-terminal domain as shown in Fig. 4A. They also described a pocket shape with the specific size of a Fuc molecule (Fig. 4B) or compounds of similar sizes and structures, such as fucosyl fluoride (Fig. 4C). They also observed that in the active site are the two catalytic residues: Asp224 and Glu266, which

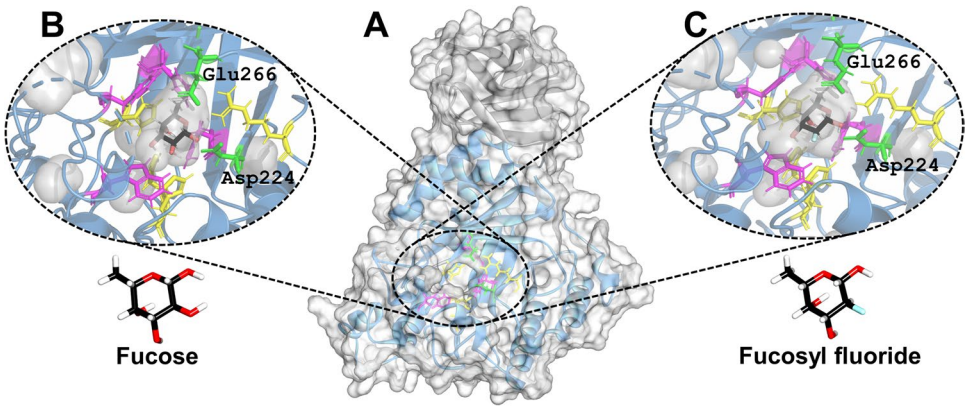


Fig. 4. (**A**) Structural model of the FUC-*Tm* in blue represents the amino acid sequence of the *N*-terminal domain. Lateral projections indicate the interactions of (**B**) Fuc (PDB 1ODU) and (**C**) fucosyl fluoride (PDB 1HL9) with amino acids in the active site (catalytic amino acids in green, aromatic in magenta, and basic in yellow). NOTE: In (**B**) and (**C**) grey spheres represent cavities within enzyme structure, such as the active site pocket

interact with the two compounds that form a complex with FUC-*Tm* as can be seen in Fig. 4B and C. Subsequently, Cobucci-Ponzano et al. (2009) and Wu et al. (2010) observed that the active site of FUC-*Tm* is surrounded by aromatic (Trp, Phe and Tyr) and (His and Arg) residues which both contribute to the binding of the substrate at the active site, which coincides with what is described by Koval'ová et al. (2019). On the other hand, to determine the strength of the binding in the formation of the substrate-enzyme complex (ES), Sulzenbacher et al. (2004) calculated the affinity of the enzyme for fucosyl fluoride, obtaining a K_m up to three times greater than that obtained for *p*NPFuc. These data show that the enzyme has a greater affinity for fucosyl fluoride than that presented by *p*NPFuc, which could be due to the inductive effect caused by the presence of an atom as electronegative as fluorine. That could affect the formation of oxocarbenium.

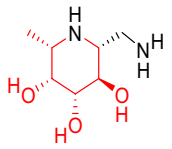
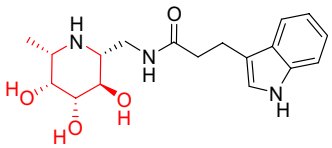
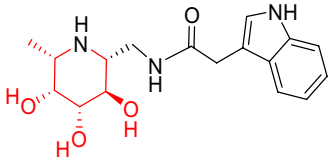
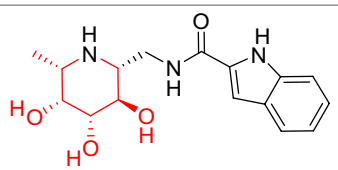
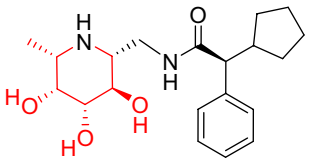
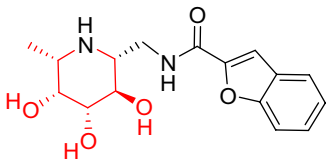
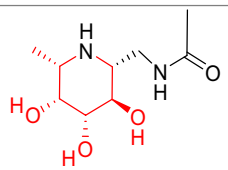
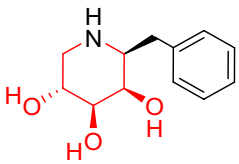
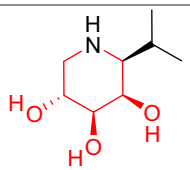
In fact, evaluating the binding affinity of different compounds that could inhibit the activity of FUC-*Tm*, along with the analysis of the way they bind to the active site, would go a long way to determine which of these inhibitors can help in the search for treatments for the various diseases associated with these enzymes. To date, a wide variety of compounds that have the potential to inhibit the activity of glycosidases in general, and FUCs in particular, have been thoroughly studied. A determining factor for inhibition activity of these compounds is the sp^2 hybridization of the anomeric carbon with which they acquire a conformation similar to that of the oxocarbenium ion, which the substrate adopts when forming the ES complex (Scheme 1). It has also been reported that there is a region outside of the active site with hydrophobic characteristics, which contributes to the greater binding force of the aglycon part of the inhibitor. The aglycon must have an aromatic ring at a distance of between 4 to 5 atoms away from the anomeric carbon, which will contribute to hydrophobic interactions in the outer region of the active site (Hattie et al. 2016; Coyle et al. 2019; Simone et al. 2022). For example, Coyle et al. (2019) reported that when modifying the oxime substituent of the *N*-phenyl carbamate PUG*N*Ac the inhibition potential can vary from 440 to 4.8 µM, observing that in the compound with the lower K_i the oxime substituent is a long chain with a phenyl group attached at the end of the chain (Coyle et al. 2019). Furthermore, until August 2022 the compound reported to inhibit FUC-*Tm* the most presents a K_i of 0.469 pM (Wu et al. 2010; Simone et al. 2022; Table 3, Entry 2).

The work carried out by Wu et al. (2010) is still one of the most interesting works on FUC inhibition so far. The authors deepen the knowledge of the way that inhibitors interact with the active site of FUC-*Tm*. The kinetic inhibition values reported for nine compounds derived from fuconojirimycin are presented in Table 3. Among the compounds evaluated, FUC-*Tm* has a greater affinity for the second compound in the Table (entry 2). Wu et al. (2010) observed that K_i is time dependent because its value decreases from 105 to 0.469 pM, the lowest value recorded so far for a fucosidase. Authors suggested that this slow-binding inhibition phenomena referred to a progressive tightening of the enzyme-inhibitor complex. This may indicate that the inhibitor undergoes conformational changes when attached to the active site.

To have a broader view of the greater affinity of compound 2 from Table 3, a computational analysis of the interaction at the active site was carried out. For this, the BIOVIA Discovery Studio Visualizer v20.1.0.19295 program and the crystallographic structure of the FUC-*Tm* (PDB 2ZX5) in complex with compound 2 were used. As can be seen in Fig. 5B, the end of the aglycon is in the outer part of the pocket of the active site and the fucosylated part is inside, coinciding with what is described by Sulzenbacher et al. (2004) for Fuc and fucosyl fluoride (Fig. 4B).

In addition, Miura et al. (2019) synthesized fluorogenic inhibitors to screen FUCA1 activity. Authors tested the affinity of the molecules through docking simulations between the inhibitors and a crystal structure of FUC-*Tm* (PDB ID: 2ZXD). They found that there was interaction between ligands and FUC-*Tm*, where fucosyl moiety accommodates at subsite -1 while the fluorogenic aglycon stands at the + 1 subsection in the active site. According to the authors + 1

Table 3 Time-dependent K_i values of compounds reported by Wu et al. (2010). In red the structural similarity with Fuc is indicated. Time dependent values were measured during a 60 min time span. *Not time-dependent value

Entry	Structure	K_i (pM)
1		1.63 x 10^{4}*
2		105 to 0.469
3		475 to 25.1
4		427 to 32.2
5		1005 to 54.2
6		441 to 231.4
7		4470*
8		5 x 10^{6}*
9		6 x 10^{6}*

region structure presents a steric impediment so compounds with a bulky aglycon end could be incompatible with this subsite. But that was not the case for compound 2, as its aglycon moiety does present these characteristics and therefore presents compatibility with the + 1 subsite. These observations agree with what is described by Coyle et al. (2019) and Simone et al. (2022) for a compound to present a high inhibition potential. Based upon the high affinity presented by compound 2 (Table 3), inhibitors designed to combat the medical conditions caused by FUCs, will need to have not only a "fucosylated" end that enters the pocket of the active site or subsite -1, which is the most common strategy, but also an aglycon compatible with the + 1 subsite.

According to the previous paragraph, it can be assumed that the inhibition potential is related not only to the interaction of the fucosylated end, but also to aglycon, in the -1 and + 1 subsites of the active site of the FUCs. For example, as can be seen in Fig. 6, when compounds in Table 3 (Entries 2 and 9) are compared, in the subsite –1 region, which is where the fucosylated group interacts, in both cases there are interactions of van der Walls type such as pi-alkyl or hydrogen-carbon as well as the formation of hydrogen bonds. In the case of subsite + 1 where the aglycon interacts, as can be seen for compound 9, this moiety is smaller compared to that of compound 2 so it has less interaction with the subsite + 1. This explains why, as can be seen in Table 3, this compound has a K_i value of six orders of magnitude higher in relation to that of compound 2 (Wu et al. 2010). Additionally, Pérez-Escalante et al. (2022) analysed the interaction of *p*NPFuc by computational methods, reporting predominantly van der Waals interactions at the active site with fewer hydrogen bonds. If these interactions are compared with those presented by compound 2 (Fig. 6), the substrates must have a lower interaction at the active site to allow hydrolysis. Inhibitors with greater binding will not allow hydrolysis. Likewise, the results of Wu et al. (2010) are comparable with what was reported by Coyle et al. (2019) who synthesized various compounds with the potential to inhibit the activity of FUCs based upon crystallographic analysis of their interaction with a FUC from *Bacteroides thetaiotaomicron*, an enzyme that has a 27% identity with human FUC.

Conclusions

When researchers think of fucosidases either as tools for preparation of FucOS or as target inhibition molecules, their attention centres on the interaction between the substrates and the residues within the active site. But the evidence gathered with the best inhibitor molecules known so far, shows that the areas next to the active site help create a better environment for the best inhibitors to bind to the

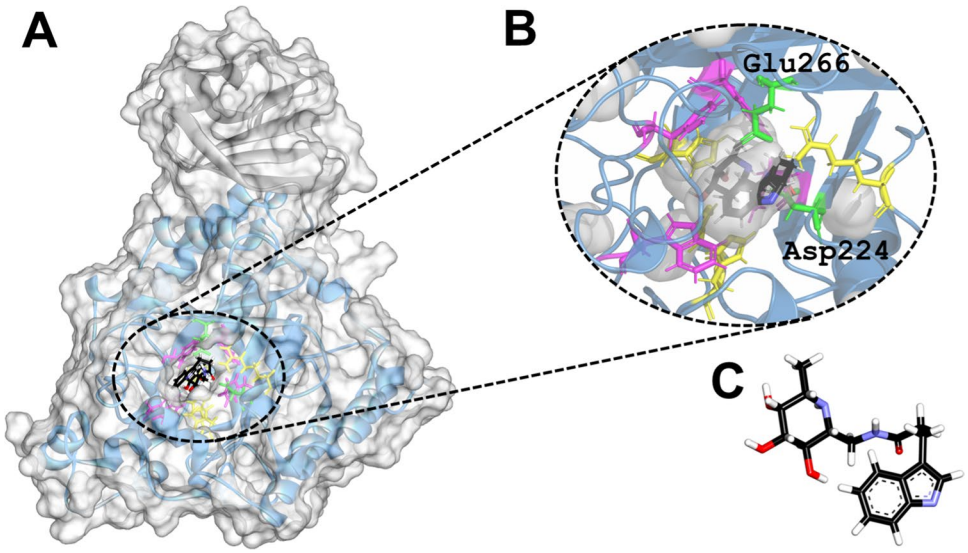


Fig. 5 Structural model of the FUC-*Tm* complex with compound 2 (Table 3) reported by Wu et al. (2010). Amino acid sequence of the *N*-terminal domain (blue), catalytic amino acids (green), aromatic (magenta), and basic (yellow). **A** Complex with compound 2 (PDB: 2ZX5). **B** Inhibitor molecule 2 located in the pocket of the active site (grey surface) of FUC-*Tm*. **C** 3D representation of compound 2

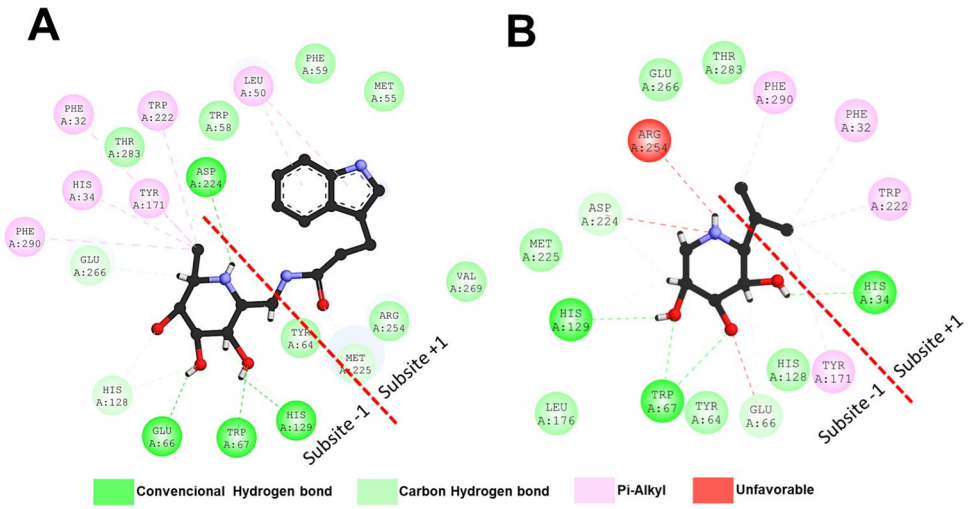


Fig. 6. 2D schematic of the interactions between FUC-*Tm* active site amino acids and piperidinylmethyl propanamide **2** (**A**) and piperidinetriol **9** (**B**) reported by Wu et al. (2010)

enzyme. Even further, the most active Fuc-donor, *p*NPFuc, presents an aglycon that interacts with the enzyme in the vicinity of the active site, although these interactions do not prevent transfucosylation. Thus, there is still more research to do in these fields to better control FUCs, either as a target or as a tool. Knowing the bases of the intermolecular interactions of FUCs and their substrates and inhibitors will help to predict the precise way in which they act. This will allow us to precisely manipulate these enzymes for the benefit of the population.

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Author contributions All authors contributed equally to the manuscript.

Declarations

Conflict of interest The authors declare no conflict of interest.

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