Probing the Acceptor Active Site Organization of the Human Recombinant $\beta1,4$-Galactosyltransferase 7 and Design of Xylose-based Inhibitors*

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Background: Glycosyltransferase inhibitors have important applications in therapeutics and as chemical biology tools.

Results: The human $\beta1,4$-galactosyltransferase 7 enzyme active site was mapped by modeling, mutagenesis, and in vitro/in cellulo assays, and novel inhibitors were synthesized.

Conclusion: An efficient inhibitor of $\beta1,4$-galactosyltransferase 7 and glycosaminoglycan synthesis was obtained.

Significance: This inhibitory molecule can be exploited to investigate glycosaminoglycan biology and modulate glycosaminoglycan synthesis in therapeutics.

Among glycosaminoglycan (GAG) biosynthetic enzymes, the human $\beta1,4$-galactosyltransferase 7 (h$\beta4GalT7$) is characterized by its unique capacity to take over xylose derivatives linked to a hydrophobic aglycone as substrates and/or inhibitors. This glycosyltransferase is thus a prime target for the development of regulators of GAG synthesis in therapeutics. Here, we report the structure-guided design of h$\beta4GalT7$ inhibitors. By combining molecular modeling, in vitro mutagenesis, and kinetic measurements, and in cellulo analysis of GAG anabolism and decorin glycosylation, we mapped the organization of the acceptor binding pocket, in complex with 4-methylumbelliferone-xylopyranoside as prototype substrate. We show that its organization is governed, on one side, by three tyrosine residues, Tyr194, Tyr196, and Tyr199, which create a hydrophobic environment and provide stacking interactions with both xylopyranoside and aglycone rings. On the opposite side, a hydrogen bond between the His195 nitrogen backbone and the carbonyl group of the coumarinyl molecule to develop a tight binder of h$\beta4GalT7$. This led to the synthesis of 4-deoxy-4-fluoroxysteryl linked to 4-methylumbelliferone that inhibited h$\beta4GalT7$ activity in vitro with a $K_i$ 10 times lower than the $K_m$ value and efficiently impaired GAG synthesis in a cell assay. This study provides a valuable probe for the investigation of GAG biology and opens avenues toward the development of bioactive compounds to correct GAG synthesis disorders implicated in different types of malignancies.

Glycosaminoglycans (GAGs) are linear heteropolysaccharide chains covalently attached to the core protein of a variety of proteoglycans (PGs). Because of their high structural diversity and their anionic characteristics, GAGs interact with a network of cellular and extracellular mediators including cytokines and chemokines, enzymes and enzyme inhibitors, matrix proteins, and membrane receptors (1). There is currently great emphasis on the crucial roles of GAGs in numerous physiological events including cell differentiation, proliferation and migration (2), and its pathological aspects, such as tumor formation, progression, and metastasis (3). Furthermore, because PGs are ubiquitously expressed in extracellular matrices and on cell surfaces of virtually every tissue, they are also involved in the normal and pathological functions of the cardiovascular and osteoarticular system (4), in amyloid disorders (5) and in axonal de- and regeneration (6). GAG biosynthesis is initiated by the formation of a tetrasaccharide linkage region (GlcA$\beta1$–3Gal$\beta1$–3Gal$\beta1$–4Xyl$\beta1$–O-) covalently linked to serine residues of the PG core protein (7). This tetrasaccharide acts as a primer for the elongation of major GAG chains, i.e. chondroitin/dermatan sulfate or heparin/heparan sulfate, which polymerization involves the coordinated activities of chondroitin-sulfate synthases and heparan-sulfate synthases (exostosins, EXT), respectively (8, 9). Mature GAG chains are finally produced by the modifications of their constitutive disaccharide units catalyzed by epimerases and sulfortransferases, which considerably increase their structural and functional diversity (10, 11).

The human xylosylprotein $\beta1,4$-galactosyltransferase (EC 2.4.1.1337, h$\beta4GalT7$) catalyzes the transfer of the first Gal res-
The importance of conserved DVD and FWGWRGE substrate binding (17, 31, 32). We previously investigated the critical active site amino acids implicated in catalysis and/or recombinant human enzyme for several years and identified been involved in structure-activity relationship studies of the enzyme. Recently, we showed that R270C replacement reduced catalytic domain, resulting in a partially or totally inactive enzyme. Based on these and previous findings, we synthesized compounds that incorporate critical structural elements both on the xylopyranoside and on the aglycone moieties to tightly bind the acceptor site of hβ4GalT7. This work revealed that the 4-deoxy-4-fluoro-Xyl linked to 4-methylumbelliferone (4-MU) strongly inhibited hβ4GalT7 activity in vitro and efficiently impaired GAG synthesis in a cell context. Such a compound will be a valuable tool for the exploration of GAG and PG synthesis and opens avenues toward the development of bioactive oligosaccharide structures for GAG biosynthesis regulation in a number of diseases implicating disorders of GAG synthesis.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—4-Methylumbelliferoyl-β-D-xylopyranoside (4-MUX), UDP-α-D-Gal (UDP-Gal), and anti-goat IgG (whole molecule) peroxidase-conjugated antibody were provided from Sigma. Anti-Myc antibodies were from Invitrogen, whereas anti-decorin antibodies were from R&D Systems. Na₂[35S]SO₄ was from PerkinElmer Life Sciences. Cell culture medium was purchased from Invitrogen and restriction enzymes, T4 DNA ligase, and peptide N-glycosidase F from New England Biolabs. The eukaryotic expression vector pcDNA3.1(+) and competent One Shot® Top 10 Escherichia coli cells were provided by Invitrogen and the bacterial expression vector pET-41a(+) and E. coli BL21(DE3) cells were from Novagen-EMD Chemicals. The QuikChange site-directed mutagenesis kit was from Stratagene and the transfection agent ExGen 500 from Euromedex.

Chemical Synthesis—Naphthyl-4-deoxy-β-D-xylopyranoside (4H-Xyl-NP) (29) was obtained after protection of the 2,3 position of naphthyl-β-D-xylopyranoside by isopropylidene acetal followed by radical deoxyanionization and deprotection. 4-Methylumbelliferonyl-4-deoxy-β-D-xylopyranoside (4H-Xyl-MU) and 4-methylumbelliferoyl-4-fluoro-β-D-xylopyranoside (4F-Xyl-MU) were synthesized from the reported starting material 4-methylumbelliferone-2,3-di-O-benzyl-β-D-xylopyranoside (35) by radical deoxyanionization or stereocontrolled 4-fluorination followed by final deprotection (data not shown).

Molecular Modeling of the hβ4GalT7 Active Site in the Presence of 4-MUX and UDP-Gal—The crystal structure of hβ4GalT7 bound to UDP and to the manganese ion (PDB code 4IRQ) was used as template (34). The crystal structure of dβ4GalT7 (PDB code 4M4K), an inactive mutant (D211N) of dβ4GalT7 in complex with UDP-Gal, and xylobiose was superposed to the human enzyme structure, which was straightfor-
ward considering their strong sequence similarity (58% overall identity). Due to crystallization conditions, a Tris molecule is bound within the active site of the hβ4GalT7. When retrieved, it frees space within the cavity that can thus accommodate the Gal moiety. The coordinates of the Gal molecule from the dβ4GalT7 complex were merged to the UDP moiety of hβ4GalT7. This did not generate any steric clash within the active site. The resulting complex was then prepared using the Protein Preparation Wizard tool of the Schrödinger Suite (Schrödinger LLC, New York), with default settings (36). All water molecules were retrieved, except the one that coordinates the manganese ion. The hydrogen atoms were added to the protein and the ligand, ascribing a pH of 7.0. The histidine residues were treated as neutral. The selection of histidine enantiomers and the orientation of the asparagine and glutamine side chains were performed so as to maximize the hydrogen bond network. The partial atomic charges derived from the OPLS-2005 force field (37) were assigned to all ligand and protein atoms. Finally, an all-atom energy minimization with a 0.3 Å heavy-atom root mean square deviation criteria for termination was performed using the Impref module of Impact and OPLS-2005 (38). The 4-MUX ligand was prepared using the ligprep module (Schrödinger Release 2014-22014). The docking program Glide was used in Standard Precision mode, with OPLS-2005, to run rigid-receptor docking calculations (39, 40). The shape and physicochemical properties of the binding site were mapped onto a cubic grid with dimensions of 20 Å centered on the xylobiose. During the docking calculations, the parameters for van der Waals radii were scaled by 0.80 for receptor atoms with partial charges less than 0.15e. Ring conformational sampling was not allowed to maintain the 4C1 conformation of the Xyl ring, and no constraint was introduced. A maximum of 100 poses were ranked according to the GlideScore scoring function. The best-docked pose of the 4-MUX ligand showed a root mean square deviation on the Xyl ring heavy atoms of 0.5 Å with the crystallographic xylobiose ligand, thus validating the docking protocol able to recover the maximum of 100 poses were retained and ranked according to the GlideScore scoring function. The best-docked pose of the 4-MUX ligand showed a root mean square deviation on the Xyl ring heavy atoms of 0.5 Å with the crystallographic xylobiose ligand, thus validating the docking protocol able to recover the position of this moiety.

**Expression Vector Construction**—The hβ4GalT7 sequence (GenBank® nucleotide sequence accession number NM_001920.3) was cloned by PCR amplification from a placenta cDNA library (Clontech), as previously described (41). For bacterial expression, a truncated form of hβ4GalT7 was expressed as a fusion protein with glutathione S-transferase (GST). The sequence lacking the codons of the first 60 N-terminal amino acids was amplified from the full-length cDNA and subcloned into NcoI and NotI sites of pET-41a (+) to produce plasmid pET-β4GalT7 (31). For the heterologous expression of hβ4GalT7 in mammalian cell lines, the full-length cDNA sequence was modified by PCR to include an AsfI site, a Kozak consensus sequence at the 5’ end, a sequence encoding a His6 tag, and an XhoI site at the 3’ end. This sequence was subcloned into pcDNA3.1 (+) to produce pcDNA-decorinHis as previously described (31).

**Expression and Purification of the Soluble Form of hβ4GalT7**—A single colony of E. coli BL21 (DE3) cells transformed with the pET-β4GalT7 plasmid was cultured overnight at 37 °C in Luria broth (LB) medium containing 50 μg/ml of kanamycin. The overnight culture was transferred into fresh LB medium (1:100 dilution), supplemented with 50 μg/ml of kanamycin, and incubated at 37 °C until the A600 value reached 0.6–0.8. Expression of hβ4GalT7 was induced by addition of 1 mM isopropyl β-D-thiogalactopyranoside to the cell suspension, which was then incubated overnight at 20 °C under continuous shaking (200 rpm). The bacterial cells were then harvested by centrifugation at 7,000 × g for 10 min at 4 °C. The pellet was resuspended in Lysis buffer (50 mM sodium phosphate, 1 mM phenylmethanesulfonyl fluoride, 1 mM EDTA, and 5% (v/v) glycerol, pH 7.4) supplemented with protease inhibitor mixture tablets (1 tablet/12 ml; Roche Diagnostics) and Benzonase® Nuclease (250 units/10 ml, Sigma). The suspended cells were then sonicated for 8 cycles of 30 s at 30% power (Badelon Sonoplus GM70) with a 20-s interval on ice between each cycle. Soluble proteins were collected from the supernatant after centrifugation for 25 min at 12,000 × g and clarification by filtration (0.2 μm Supor® Membrane; PALL-Life Science). 10 ml of clarified extracts were applied onto a 1–ml glutathione-Sepharose High Performance column (GSTrap HP; GE Healthcare) connected to an AKTA prime plus instrument (GE Healthcare). Protein was eluted as 1-ml fractions using 50 mM Tris-HCl, pH 8.0, containing 10 mM reduced glutathione buffer. Protein purity of the eluted fractions was evaluated by 12% (w/v) SDS-PAGE analysis, followed by staining with Coomassie Brilliant Blue. Fractions containing the pure protein were used to determine the kinetic parameters of the enzyme. The same procedure was used for purification of the mutants. Protein concentration was measured using Quant-iT™ assay kit and Qubit™ spectrophotometer.

**Determination of the In Vitro Kinetic Parameters of hβ4GalT7**—The kinetic parameters kcat and Km toward 4-MUX and UDP-Gal were determined as described (31). Briefly, 0.2 μg of purified wild-type or mutated GST-hβ4GalT7 were incubated for 30 min at 37 °C in a 100 mM sodium cacodylate buffer, pH 7.0, 10 mM MnCl2, with concentrations from 0 to 5 mM 4-MUX in the presence of fixed 1 mM UDP-Gal to determine the apparent Km toward 4-MUX, and with concentrations from 0 to 5 mM UDP-Gal in the presence of fixed 5 mM 4-MUX to determine the apparent Km toward UDP-Gal. The incubation mixture was then centrifuged at 10,000 × g for 10 min at 4 °C. The supernatant was analyzed by high performance liquid chromatography (HPLC) with a reverse phase C18 column (xBridge, 4.6 × 150 mm, 5 μm, Waters) using Waters equipment (Alliance Waters e2695) coupled to a UV detector (Shimadzu SPD-10A). Kinetic parameters were determined by
nonlinear least squares regression analysis of the data fitted to
the Michaelis-Menten rate equation using the curve-fitter pro-
gram of Sigmaplot 9.0 (Erkraft, Germany).

In Vitro Competition Assays of hβ4GalT7 Activity by
C4-modified Xylosides—The in vitro inhibition assays of the
wild-type GST-hβ4GalT7 were carried out using 0.2 μg of puri-
ified protein incubated for 30 min at 37 °C in a 100 mM sodium
cacodylate buffer, pH 7.0, 10 mM MnCl2, with 0.5 mM 4-MUX
and 1 mM UDP-Gal, in the presence of concentrations from 0 to
5 mM 4H-Xyl-NP, 4H-Xyl-MU, or 4F-Xyl-MU. Quantification
of the reaction product was carried out by HPLC, as described
above. The enzyme activities were reported as a function of the
logarithmic values of inhibitor concentration. IC50 values were
determined by fitting the experimental dose-response curves
using the curve-fitter program of Sigmaplot 9.0 (Erkraft, Ger-
many). K values were calculated from IC50 values according to
the Cheng-Prusoff’s equation (42, 43).

In Cellulo Analysis of GAG Chains Biosynthesis by
Na2[35SO4] Incorporation—GAG chains biosynthesis using
4-MUX as primer substrate was determined with CHOpgsB-618
cells (American Type Culture Collection). Cells were cul-
tured in Dulbecco’s modified Eagle’s medium/F-12
(DMEM/F-12) (1:1), supplemented with 10% fetal bovine
serum (Dutsch), penicillin (100 units/ml)/streptomycin (100
mg/ml), and 1 mM glutamine, then transfected with the wild-
type or mutant pcDNA-β4GalT7-Myc plasmid or with the empty
pcDNA3.1 vector at 70% cell confluency. Transfected cells were
then incubated in low sulfate medium (Fisher) sup-
plemented with 10 μCi/ml of Na2[35SO4] in the presence of 0.5
or 10 μM 4-MUX for 16 h. For GAG chain isolation 1 ml of
culture medium was applied to a G-50 column (GE Healthcare)
to separate radiolabeled GAG chains from the non-incorpo-
rated Na2[35SO4] and radiolabeling was quantified by scintilla-
tion counting. In parallel, the hβ4GalT7 expression level was
checked by Western blotting using a primary anti-Myc
(1/5,000) and a secondary anti-mouse antibody (1/10,000). To
test the inhibitory potency of C4-modified xylosides, the mole-
cules were added at 0 to 100 μM concentrations together with
4-MUX (5 μM) for 16 h prior to isolation and quantification of
radiolabeled GAGs. To test the cytotoxicity of xylose inhibi-
tors in CHOpgsB-618 cells expressing the wild-type hβ4GalT7,
cells were seeded at 150,000 cells/well in 12-well plates, and
incubated for 48 h at 37 °C in the presence of 0 to 400 μM
inhibitor, or 4-MUX as a control. The ratio of viable cells upon
the total number of cells was determined using the cell counter
TC20 (Bio-Rad) in the presence of a vital marker (trypan blue).

In Cellulo Analysis of Decorin Core Protein Glycosylation—
CHOpgs-B618 cells stably transfected with pcDNA-decorinHis
encoding the human decorin core protein (31, 44) were tran-
siently transfected with pcDNA3.1 or with recombinant vector
encoding either the wild-type or mutated hβ4GalT7-Myc as
described above. 48 Hours following transfection, the cell
medium was collected, concentrated by centrifugation at 4 °C
for 15 min at 3000 × g, using the Amicon Ultracell 30 MWCO
concentrating system (Merck Millipore, Germany), and sub-
tmitted to SDS-PAGE (25 μg of protein/well). The glycosylation
level of the decorin core protein was monitored by immunoblot
using a 1/5,000 dilution of primary polyclonal anti-human
decorin antibody (VWR) and a 1/10,000 dilution of secondary
anti-goat antibody coupled to horseradish peroxidase (Sigma),
then quantified using Image) software. Briefly, the level of
decorin glycosylation was expressed as relative band intensity
by normalizing the band intensity value for the glycosylated
form upon the total intensity value for the bands corresponding
to the glycosylated and non-glycosylated forms of decorin core
protein. The expression level of the decorin core protein in
pcDNA3.1-transfected cells was used as the negative control
and served as a loading control. On the other hand, the level of
glycosylated decorin in cells expressing wild-type hβ4GalT7
was used as positive control for decorin glycosylation.

RESULTS

Molecular Modeling of the hβ4GalT7 Acceptor Binding Site—
In the present study, we aimed to identify amino acids impor-
tant for structural organization of the hβ4GalT7 acceptor sub-
strate binding site. We took advantage of the recent crystal
structure of hβ4GalT7 in complex with UDP (34) to build a
molecular model of this enzyme in complex with both the sugar
donor UDP-Gal and the acceptor 4-MUX (Fig. 1A). The mod-
eled structure is in a closed conformation, considered to be the
catalytically competent form, and the hydrogen bond network
around the UDP moiety is fully conserved. We first examined
the position of a series of tyrosine residues, i.e. Tyr194, Tyr196,
and Tyr199, that were suggested to be involved in binding of the
xylobiose in the dβ4GalT7 structure (33). Our computational
analysis indicates that Tyr194 stabilizes both the donor and
acceptor substrates location by establishing a hydrogen bond
with a β-phosphate oxygen of UDP-Gal and a π-stacking inter-
action with the 4-methylumbelliferyl moiety, respectively (Fig.
1A). Residue Tyr196 is not hydrogen bonded to the substrates
but to the side chain of residue Asp229, allowing its second car-
boxylic oxygen to be suitably oriented to form a hydrogen bond
with the O2 atom of Xyl. The spatial orientation of Tyr199 inside
the substrate binding pocket allows the formation of a hydro-
gen bond between its side chain hydroxyl and the O2 atom of
the Gal moiety of UDP-Gal. Altogether, residues Tyr194, Tyr196,
and Tyr199 form a strongly hydrophobic cluster that is required
for correct binding of the substrates.

Analysis of the His195 position, a conserved amino acid
located between the two active site Tyr194 and Tyr196 residues,
shows no hydrogen bond involving its side chain. However, the
backbone nitrogen atom of this residue is hydrogen bonded with
the CO group of 4-MUX (Fig. 1A). As illustrated in Fig. 1B,
Arg226 is located on the surface of the acceptor binding site
contributing to an amphipathic entry door with the aromatic
residues. In our model, there is no hydrogen bond involving
the side chain of Arg226. Instead, its backbone nitrogen atom is
hydrogen bonded with the O3 atom of the Xyl moiety of
4-MUX (Fig. 1A).

The structural impact of Arg270 on enzyme activity, in the
context of EDS was also addressed. The model structure of
hβ4GalT7 reveals that Arg270 belongs to the flexible loop (261–
284) that moves upon donor substrate binding, thus creating
the acceptor substrate binding site (Fig. 1A). This conforma-
tional change leads to the closed and catalytically competent
conformation of the active site. However, the crystal structure
of the human enzyme (34), as well as our own model in complex with both the donor and acceptor substrates do not highlight specific interactions established by this residue, although its close location to the surface of the active site has to be underlined (Fig. 1B).

**Kinetic Properties of the Human Recombinant hβ4GalT7 Mutants Expressed in E. coli**—To assess the functional importance of the residues of the acceptor binding site highlighted by our model, we carried out point mutagenesis and analyzed the consequences of conservative and non-conservative mutations on the kinetic parameters of hβ4GalT7 expressed and purified from recombinant E. coli cells. The wild-type enzyme and engineered mutants were produced as truncated fusion proteins lacking the 60 N-terminal amino acids (including the transmembrane domain and part of the stem region) linked to GST and purified by affinity chromatography (data not shown). This led to 1.0 to 2.5 mg of pure protein per liter of culture for wild-type and mutant hβ4GalT7. Kinetic assays were performed using 4-MUX as acceptor substrate, which allowed quantification of the transfer reaction product by UV detection coupled to HPLC. The \( k_{cat} \) and \( K_m \) values of the wild-type enzyme toward UDP-Gal and 4-MUX shown in Table 1 were in agreement with previous work (17, 31).

Substitution of Tyr\(^{194}\) by alanine led to an inactive enzyme, and its conservative substitution by phenylalanine did not restore the galactosyltransferase activity of hβ4GalT7 (Table 1), indicating a critical role of this residue and, importantly, of the hydroxyl group of the tyrosine side chain. The mutation of
Kinetic parameters of wild-type and mutant GST-β4GalT7

TABLE 1

| Enzyme          | $k_{\text{cat}}$ | $K_m$  | $k_{\text{cat}}/K_m$ | $K_m$  | $k_{\text{cat}}/K_m$ |
|-----------------|------------------|--------|----------------------|--------|----------------------|
|                 | \( \text{min}^{-1} \) | \( \text{mM} \) | \( \text{min}^{-1} \text{mM}^{-1} \) | \( \text{mM} \) | \( \text{min}^{-1} \text{mM}^{-1} \) |
| GST-β4GalT7     | 90.5 ± 2.3       | 0.22 ± 0.02 | 425 | 0.35 ± 0.02 | 220 |
| Y194A           | ND               | ND      | ND                   | ND     | ND                   |
| Y194F           | ND               | ND      | ND                   | ND     | ND                   |
| H195A           | 115.9 ± 9.7\(^b\) | 0.40 ± 0.02 | 291 | 0.64 ± 0.02\(^b\) | 180 |
| H195Q           | 97.4 ± 2.4\(^b\) | 0.33 ± 0.02\(^a\) | 295 | 0.55 ± 0.02\(^b\) | 177 |
| H195R           | 89.6 ± 1.3       | 0.32 ± 0.02\(^a\) | 295 | 0.35 ± 0.03 | 242 |
| Y196A           | ND               | ND      | ND                   | ND     | ND                   |
| Y196F           | 30 ± 1.1\(^a\)  | 0.34 ± 0.06\(^b\) | 88 | 1.06 ± 0.06\(^b\) | 28 |
| Y199A           | ND               | ND      | ND                   | ND     | ND                   |
| Y199F           | 72.3 ± 5.8\(^b\) | 0.32 ± 0.02\(^b\) | 243 | 0.59 ± 0.06\(^b\) | 113 |
| R226A           | 53.6 ± 2.0\(^b\) | 0.34 ± 0.03\(^a\) | 171 | 0.44 ± 0.05\(^a\) | 112 |
| R226K           | 81.1 ± 2.9\(^b\) | 0.29 ± 0.02\(^b\) | 282 | 0.46 ± 0.01\(^b\) | 175 |
| R270A           | 46.4 ± 0.2\(^b\) | 0.27 ± 0.01\(^b\) | 184 | 0.60 ± 0.02\(^b\) | 72 |
| R270K           | 48.7 ± 1.5\(^b\) | 0.37 ± 0.02\(^b\) | 139 | 0.54 ± 0.07\(^b\) | 85 |

\(^a\) ND indicates that no kinetic constant could be determined using excess acceptor or donor substrate.

\(^b\) The results were analyzed with Student’s t test and considered as significant when $p < 0.05$.

Tyr\(^{196}\) to alanine totally abolished enzyme activity, whereas replacement of this residue by phenylalanine led to a slightly active enzyme. The Y196F mutation did not impair enzyme affinity toward the donor substrate to a major extent but this mutant presented a lower affinity toward 4-MUX with a $K_m$ value about 3-fold that of the wild-type enzyme (Table 1). As observed in the case of Tyr\(^{194}\) and Tyr\(^{196}\), the non-conservative mutation Y199A led to a total loss of enzyme activity. However, similarly with what was observed for Tyr\(^{196}\), substitution of His\(^{195}\) by alanine, glutamine, or arginine was carried out. The $K_m$ values of all three mutants toward UDP-Gal and 4-MUX were mostly comparable with those of the wild-type enzyme, indicating that these mutations had no major effect upon hβ4GalT7 affinity toward its substrates. Moreover, the substitutions at position 195 did not affect the rate of reaction transfer, as indicated by the $k_{\text{cat}}$ values that were essentially unchanged (Table 1). Altogether, these results indicate that the side chain of His\(^{195}\) does not play a critical role in xyloside binding and hβ4GalT7 catalytic activity. Substitution of Arg\(^{226}\) by alanine or lysine did not impair the affinity toward the substrates with $K_m$ values for UDP-Gal and 4-MUX, which were in the same range to that of the wild-type enzyme, and produced a moderate decrease (about 2-fold) of the catalytic constant value (Table 1).

The Arg\(^{270}\) residue is mutated to cysteine in the progeroid form of the EDS syndrome, and we previously showed that this mutation led to a significant decrease in hβ4GalT7 activity, mainly due to a reduced affinity toward 4-MUX (about 10-fold, see Ref. 17). To ascertain the contribution of this residue in hβ4GalT7 activity and xyloside binding, we performed kinetic assays following the conservative R270K and non-conservative R270A mutations. The $k_{\text{cat}}$ values for both mutants were about two times lower than that found for the wild-type enzyme and the $K_m$ value toward UDP-Gal was almost unaffected (Table 1).
Structure-guided Inhibitors of hβ4GalT7

Upon addition of peptide of N-glycosidase F (PNGase F) the immunoblot analysis indicates that the mutated enzymes were all expressed at a comparable level to that of the wild-type hβ4GalT7 (Fig. 2). These results are in line with the reduced efficiency exhibited about half of that observed with cells expressing wild-type enzyme (Fig. 2). These results indicate that the GAG synthesis rate in cells expressing the R270A mutant was about 2–3-fold lower than that of the wild-type enzyme (Fig. 2). In addition, replacement of Arg226 by lysine slightly increased the GAG expression level compared with alanine substitution, reaching about 60% that obtained with the wild-type, at 5 and 10 μM 4-MUX. Corroborating in vitro kinetic parameters, these cellular assays indicate that modification of the side chain of Arg226 produces minor effects on galactosyltransferase activity.

We finally examined the impact of mutations of the Arg270 residue upon GAG synthesis in eukaryotic cells. We observed that the GAG synthesis rate in cells expressing the R270A mutant was about 55% lower than that of the wild-type enzyme, at 5 and 10 μM 4-MUX (Fig. 2). The GAG synthesis level of the conservative mutant R270K was also about 2-fold reduced compared with the wild-type (Fig. 2). These results confirm that mutations of Arg270 significantly affect the capacity of hβ4GalT7 to synthesize GAG chains from 4-MUX in a cellular context.

Effect of Tyr194, Tyr196, His195, Arg226, and Arg270 Mutations on the Ability of hβ4GalT7 to Initiate the Glycosylation of the Decorin PG in Cellulo—We next determined whether the mutations would affect GAG chain formation on the core protein of decorin, used as a model PG (31). To this aim, CHOpgsB-618 cells were engineered to stably express the recombinant human decorin, and were transiently transfected with a pcDNA3.1 vector encoding the wild-type or mutant forms of hβ4GalT7. This allowed monitoring of the GAG synthesis in the absence of 4-MUX.

In the presence of 4-MUX, the GAG synthesis rate in cells expressing H195A, H195Q, and H195R mutants was moderately reduced, i.e. 10 to 15% lower than that of cells expressing the wild-type enzyme (Fig. 2). These results indicate that the side chain of this residue does not influence galactosyltransferase activity of hβ4GalT7 in the context of 4-MUX-primed GAG chains in eukaryotic cells, corroborating the findings that none of the mutations of His195 significantly affect in vitro activity (Table 1). The level of [35SO4] incorporation in the presence of 4-MUX in cells expressing R226A was about 2 times lower than that of the wild-type enzyme (Fig. 2). In addition, replacement of Arg226 by lysine slightly increased the GAG expression level compared with alanine substitution, reaching about 60% that obtained with the wild-type, at 5 and 10 μM 4-MUX. Corroborating in vitro kinetic parameters, these cellular assays indicate that modification of the side chain of Arg226 produces minor effects on galactosyltransferase activity.

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We finally examined the impact of mutations of the Arg270 residue upon GAG synthesis in eukaryotic cells. We observed that the GAG synthesis rate in cells expressing the R270A mutant was about 55% lower than that of the wild-type enzyme, at 5 and 10 μM 4-MUX. Corroborating in vitro kinetic parameters, these cellular assays indicate that modification of the side chain of Arg226 produces minor effects on galactosyltransferase activity.
We also assessed the role of the His\textsuperscript{195} residue in the glycosylation process of decorin. The results shown in Fig. 3 indicate that none of the mutations, H195A, H195Q, or H195R, significantly affected the level of decorin glycosylation. These data are consistent with the results obtained on the in vitro and in cellulo activity of the enzyme toward 4-MUX. Together, mutagenesis experiments indicate that modification of the amino acid side chain at position 195 did not greatly affect xyloside binding and galactosyltransferase activity of h\textbeta\textbeta 4GalT7. Investigation of the effect of the Arg\textsuperscript{270} mutation upon the ability of CHOpgsB-618 cells to glycosylate decorin showed that the glycosylation level reached with cells expressing R270A or R270K mutant was about half of that obtained with cells expressing the wild-type h\textbeta\textbeta 4GalT7, consistent with in vitro and in cellulo galactosyltransferase assays (Fig. 3).

Xyloside Inhibitors Design and in Vitro and in Cellulo Competition Assays—We next took advantage of the knowledge gained from our investigation of the organization of the acceptor substrate binding site to synthesize and test xyloside analogs as potential inhibitors of h\textbeta\textbeta 4GalT7. To this end, in vitro competition assays were performed as described under “Experimental Procedures.” The specific activity as a function of the logarithm values of the inhibitor concentrations are reported in Fig. 4B and data fitted to the logistic equation provided IC\textsubscript{50} values as reported in Table 2.

Because the C4-position is critical for both binding and transfer of the Gal residue from UDP-Gal onto the xyloside acceptor, we first synthesized a 4-deoxy derivative of 4-MUX (4H-Xyl-MU, Fig. 4A) and tested this compound as inhibitor of h\textbeta\textbeta 4GalT7 in vitro. 4H-Xyl-MU was able to inhibit up to 50% of the initial activity at a 2 mM concentration (Fig. 4B), with an IC\textsubscript{50} value of about 1 mM and a K\texttext{f} value of about 0.5 mM (Table 2). To test whether hydrogen bond formation between 4-MUX and the protein via His\textsuperscript{195} is important for the inhibitory potency, we synthesized 4H-Xyl-NP, which the aglycone struc-
Structure-guided Inhibitors of hβ4GalT7

A chemical structures of the xyloside analogs synthesized and tested as inhibitors. From left to right: 4H-Xyl-NP, 4H-Xyl-MU, and 4F-Xyl-MU. B, inhibition assays using purified recombinant wild-type hβ4GalT7 in the presence of fixed 4-MUX (0.5 mM) and UDP-Gal (1 mM). Activities are presented as function of the logarithm of increasing inhibitor concentrations (0–5 mM): 4H-Xyl-NP (A), 4H-Xyl-MU (B), and 4F-Xyl-MU (C). Results are the mean ± S.E. of three independent determinations on assays performed in duplicate.

FIGURE 5. Inhibitory effect of C4-modified xylosides upon 4-MUX-primed GAG chains synthesis in CHOpgsB-618 cells expressing the recombinant wild-type hβ4GalT7. CHOpgsB-618 cells transiently transfected with wild-type hβ4GalT7 cDNA were incubated with 5 μM 4-MUX and Na₂[35SO₄]₂, in the presence of 4H-Xyl-NP (panel A), 4H-Xyl-MU (panel B), and 4F-Xyl-MU (panel C). The GAG expression level in cells transfected by the empty pcDNA vector was taken as negative control. Results are the mean ± S.E. of three independent experiments performed in triplicate. Statistical analysis was carried out using the Student’s t test with **, p < 0.01 and ***, p < 0.001 versus GAG synthesis rate in the absence of inhibitor.

TABLE 2
Kinetic inhibition parameters of hβ4GalT7 with C4-modified xylosides

| Xylosides     | IC₅₀   | Kᵢ   |
|---------------|-------|------|
| 4H-Xyl-NP     | ND    | ND   |
| 4H-Xyl-MU     | 1.28 ± 0.22 | 0.53 ± 0.10 |
| 4F-Xyl-MU     | 0.06 ± 0.02 | 0.03 ± 0.01 |

* ND, not determined.

To complement the in vitro assay, we assessed the ability of the synthesized xyloside derivatives to inhibit GAG chain biosynthesis in cellulo. Addition of 4H-Xyl-NP produced a moderate but significant 20% decrease of GAG chain synthesis in CHOpgsB-618 cells, when used at 50 and 100 μM (Fig. 5A). This correlated with the weak in vitro inhibition level obtained with this compound. The compound 4H-Xyl-MU allowed a larger inhibition of GAG chain synthesis, with up to 30% reduction of the GAG synthesis rate at similar concentrations (Fig. 5B). The best inhibitory effect was observed when performed in the presence of 4F-Xyl-MU (Fig. 5C). This compound produced a decrease of hβ4GalT7 activity toward 4-MUX less than 25% at the highest concentration (Fig. 4B), with an IC₅₀ of 0.06 mM and a Kᵢ of 0.02 mM. The inhibition constant for this compound is more than 10 times lower than that reached for the deoxy analog (Table 2).
ence of 4F-Xyl-MU leading to up to 50% inhibition of the initial GAG chain synthesis rate at 100 μM concentration (Fig. 5C). Interestingly, preliminary results indicated that 400 μM 4F-Xyl-MU inhibited the initial decorin glycosylation rate by about 50%, without affecting the viability of CHOpgsB-618 cells (data not shown). Altogether, the latter data confirmed that this fluorinated compound should be considered as a promising non-cytotoxic xyloside-based inhibitor of hβ4GalT7.

**DISCUSSION**

hβ4GalT7 is a unique enzyme in the GAG biosynthetic pathway with regard to its capacity to use exogenous xyloside molecules as substrates and/or inhibitors that can efficiently modulate GAG synthesis in vitro and in vivo (19, 20, 45). This glycosyltransferase is also central in the GAG synthesis process because the formation of the tetrasaccharide linker is a prerequisite to the polymerization of both heparan sulfate and chondroitin/dermatan sulfate chains. The human enzyme thus represents a prime target for the design of effectors of GAG synthesis as drugs to correct GAG disorders associated with numerous malignant conditions such as genetic diseases and cancer. To meet this challenge, we pioneered structure-function studies of the recombinant hβ4GalT7. We previously carried out structural, thermodynamic, and phylogenetic investigations that identified key amino acid residues mainly implicated in the recognition and binding of the donor substrate (31, 46). We also provided insight into the molecular basis of the GAG defects characterizing rare forms of EDS syndrome (17, 47). In the present work, to develop xyloside compounds that will specifically target the hβ4GalT7 activity for a therapeutic purpose, we explored the architecture of the acceptor substrate binding site. To this aim, we combined functional investigations including site-directed mutagenesis, kinetic analyses, in vitro and in cellulo evaluation of galactosyltransferase activity and GAG synthesis, and a computational approach. This allowed mapping the acceptor binding site and to design and synthesize a potent xyloside-based inhibitor of GAG synthesis.

We first targeted a set of three tyrosine residues, Tyr194, Tyr196, and Tyr199, as well as His195 belonging to the same conserved motif, that occupy a strategic position surrounding the xyloside acceptor substrate (Ref. 34 and the present data). Our mutational analysis led to a remarkable observation because alanine substitution of each of these tyrosines completely abolished hβ4GalT7 activity. The tyrosine-alanine mutants (i) were devoid of galactosyltransferase activity in vitro, (ii) were unable to prime GAG synthesis from 4-MUX in cellulo, and (iii) did not promote decorin glycosylation, thus supporting a prominent function of this set of aromatic residues. Mutating Tyr194, Tyr196, and Tyr199 with phenylalanine revealed that the substitution differently affected hβ4GalT7 activity depending on the position. Noteworthy, the presence of the hydroxyl group of Tyr194 was indispensable because the conservative Y194F mutant completely lacked in vitro or in cellulo galactosyltransferase activity toward 4-MUX and was unable to sustain glycosylation of decorin. This observation is likely to be explained by a functionally important interaction between the hydroxyl group of this tyrosine and the β-phosphoryl group of UDP-Gal that was observed in all structures and models of β4GalT7 (Ref. 34 and this report, see Fig. 1). Most importantly, our computational model and experimental data suggested that the critical role of Tyr194 also arises from a stacking interaction between the aromatic ring of this residue and the 4-methylumbelliferyl moiety of 4-MUX. Altogether, these data indicate that Tyr194 occupies a strategic location in the catalytic center and interacts with both the donor nucleotide and the aglycone group of 4-MUX. In the case of Tyr196, the presence of the hydroxyl group of the tyrosine residue was also a major structural element because its replacement by phenylalanine only slightly restored the activity toward 4-MUX in vitro and in cellulo. The Y196F mutant did not sustain decorin glycosylation, also supporting an important role of this residue in the glycosylation of endogenous proteoglycans. Our model provides a molecular explanation to these results, because it shows that Tyr196 is not directly involved in the binding of the acceptor substrate but rather forms a hydrogen bond between its hydroxyl group and Asp229, this latter residue establishing an important interaction with O2 of the Xyl moiety. This supports the idea that interactions with the hydroxyl in the C2 position control a strict physiologically important regulatory role of Xyl-phosphate substitution in position 2 on GAG synthesis (41, 48). Furthermore, our model suggests that this residue is part of the cavity floor in agreement with structural data indicating that the acceptor substrate xylose moiety is located in a hydrophobic binding pocket formed by Tyr177, Tyr179, and Trp207 in the Drosophila structure, and by Tyr194, Tyr196, and Trp224 in hβ4GalT7. The present data complements our previous findings demonstrating that Trp224 is a crucial active site residue (31). Differently to the preceding studied tyrosines, hβ4GalT7 tolerated well the substitution of tyrosine to phenylalanine at position 199 leading to a mutant that was active toward 4-MUX in vitro and was able to prime GAG synthesis from 4-MUX and onto the decorin core protein. Consistently, Tyr199 is substituted by a phenylalanine in the Drosophila enzyme, suggesting that the presence of a hydrophobic aromatic ring is sufficient at this position. Further analysis of our molecular model predicts hydrogen bond formation between the tyrosine hydroxyl group of Tyr199 and O2 of the Gal moiety of UDP-Gal. However, no significant change in the Km value toward UDP-Gal was observed for the Y199F mutant, indicating that this interaction may not play a critical functional role in nucleotide binding. The location of Tyr199 favors a role as contributor to the hydrophobic surrounding of the acceptor substrate binding pocket together with Tyr194, Tyr196, and Trp224 residues when hβ4GalT7 is in its closed conformation (34).

Investigation of the structural role of His195 led to the most interesting findings for the design of efficient substrates and inhibitors of hβ4GalT7. We predicted that the nitrogen atom of the peptide backbone of this residue forms a hydrogen bond with the carbonyl group of the coumarin moiety of 4-MUX. This is in full agreement with our mutational study that showed no major effect upon changing the side chain of the histidine residue at this position by alanine, glutamine, or arginine substitution on the hβ4GalT7 activity monitored in vitro and in cellulo. However, the functional importance of an interaction
between the His195 backbone and 4-MUX was clearly empha-
sized by the stronger inhibitory effect of 4-deoxy-Xyl-MU com-
pared with 4-deoxy-Xyl-NP. We also found that 4-MUX was
used as a substrate with a better affinity than Xyl-NP (data not
shown). Noteworthy, among a series of naphthyl xylosides,
Siegbahn et al. (29) showed that 6-hydroxy-naphthyl-Xyl was
able to prime GAG chains more efficiently than any other
unsubstituted derivative in breast cancer cell lines. Altogether,
this gives strong evidence that His195 through its backbone pro-
vides an important structural element for efficient binding of
4-MUX derivatives, and offers a molecular explanation for the
superiority of 4-MUX synthesized in this study over naphthyl
and benzyl-substituted xylosides previously reported in the lit-
erature (25, 30).

We also discovered a unique active site basic residue, i.e.
Arg226. Interestingly, this residue is located between the ar-
omatic-rich sequence, containing Trp224 that interacts with the β-phosphate O-atom of the donor substrate, and the sequence containing acidic residues that are involved in Xyl binding and in the transfer reaction (31). Our functional analysis showed that modifying the side chain of Arg226 by site-directed mutagenesis did not affect enzyme affinity toward acceptor or donor substrate. This is in line with the computational analysis indicating that the nitrogen atom of the peptide backbone of Arg226, but not its side chain, interacts with the O3 atom of the Xyl moiety. Fig. 1, A and B, clearly shows that this residue, together with Trp224, are brought close to the aromatic triad in the closed conformation of hβ4GalT7.

We also investigated the role of Arg270, which substitution by a cysteine residue is implicated in the progeroid form of EDS (49). Our previous studies revealed that this genetic mutation dropped in vitro hβ4GalT7 activity and impaired GAG chains synthesis in CHO-pgsB618 cells (17). These effects were sug-
gested to be due to a loss of hydrogen bonding between the lateral chain of Arg270 and the hydroxyl group of a serine residue from the PG core protein (17). This idea was supported by later molecular modeling indicating that Arg270 borders the catalytic site of hβ4GalT7 in the closed conformation (Ref. 34 and this work, see Fig. 1A). However, the precise mechanism by which Arg270 modulates in vitro and ex vivo hβ4GalT7 activities remains unclear. Indeed, current crystal structures and molecular models do not point to a specific role of this residue in catalysis or substrate binding, consistent with kinetic data showing that mutations of Arg270 in alanine and lysine moderately affect hβ4GalT7 activity and affinity, and the observation that in Drosophila, the corresponding position is occupied by a lysine residue. The Arg270 residue is located in the flexible loop (261–284) that moves upon donor substrate binding, thus creating the acceptor substrate binding site. This conformational change leads to the closed and catalytically competent conformation of the active site. It thus can be expected that any mutations affecting the loop movement would impair the transfer reaction. However, why the substitution of Arg270 by a cysteine residue that causes the progeroid form of EDS patients, pro-
duces more deleterious consequences than alanine or lysine
mutations requires further investigation of the conformational modifications operating during the catalytic cycle.

Our current and previous functional and computational approaches provide a detailed cartography of the hβ4GalT7 acceptor substrate binding pocket for the rational design of xyloside-based inhibitors (31). We show that the active site organization is governed, on one side, by a series of aromatic amino acids comprising three Tyr residues, i.e. Tyr194, Tyr196, and Tyr199, which together with Trp224 create a hydrophobic environment and provide stacking interactions essential to the binding of both the xylosyl and aglycone parts of the acceptor substrate. On the opposite side of the site, it involves a network of hydrogen-bonding interactions between three charged amino acids, i.e. Asp228, Asp229, and Arg226, and the hydroxyl groups of the Xyl moiety and other active site residues. Until now, most studies aiming to inhibit cellular and extracellular GAG synthesis have been targeted to the synthesis and testing of xyloside derivatives acting as substrates of hβ4GalT7 thus reducing the glycosylation of endogenous proteoglycan core proteins (19). This approach successfully provided promising pharmacological agents, in particular anti-tumor compounds (45, 50). However, because such molecules behave both as exogenous primers of GAG synthesis and inhibitors of endogenous GAG formation, deciphering their mechanism of action remains challenging. With the perspective of designing xylo-
side derivatives that selectively act as inhibitors of GAG forma-
tion, we opted for C4-modified analogs, whose modification at the C4 position prevents the catalytic transfer, and first synthesized deoxy derivatives. In addition, we took advantage of the information gained from our structural and mutational analyses. We considered two key elements of the aglycone binding, i.e. the strategic position of Tyr194 that forms stacking interactions with the aglycone part of the acceptor substrate as well as the interaction between His195 N-backbone and the carbonyl group of the coumaryl moiety. In agreement with our predic-
tion, our results clearly show that the 4-deoxy-xyloside appended to 4-MU was superior to the naphthyl-substituted molecule, as indicated by in vitro and in cellulo studies, sup-
porting the idea that the hydrogen bond between His195 and the carbonyl group of the coumaryl group is crucial. Interestingly, Tsuchiuchi et al. (28) found that among triazole xyloside deriva-
tives generated by click chemistry bearing various aromatic and nonaromatic aglycones, the p-nitrophenyl analog was the best inhibitor of PG synthesis when screened in endothelial cells. Although detailed docking of these triazole derivatives should be performed, it is tempting to speculate that the formation of a hydrogen bond interaction between the aglycone nitro group and His195 enhances the inhibitory potential compared with the other substituted benzyl derivatives. Furthermore, we show here that the 4-deoxy-4-fluorinated 4-MUX was superior to the unsubstituted 4-deoxy analog, indicating that addition of an electronegative atom at this position is an important element in the design of potent inhibitors. The 4-hydroxy group is involved in two hydrogen bonds with the carboxyl group of Asp228 and the 4-hydroxy group of Gal, respectively. The replacement of the hydrogen donor hydroxyl group by a fluo-
rine atom that is larger than hydrogen and which van der Waals radius and electronegativity are closer to oxygen, at the C4 posi-
tion, enhanced binding interactions with hβ4GalT7. This cor-
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Structure-guided Inhibitors of hβ4GalT7

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xylosides act as “good” substrates or inhibitors of GAG synthesis (18, 29, 30). In the same way, fluorinated thrombin inhibitors showed improved factor Xa binding (51). Further docking calculations of fluoro-substituted xylosides are underway to assess the mechanism underlying the improved interactive properties upon fluorine incorporation.

In summary, we developed a powerful approach for the design of xyloside inhibitors that specifically target hβ4GalT7. By integrating structural elements important for the binding of both the Xyl moiety and the hydrophobic aglycone, we synthesized a xylose-based inhibitor of hβ4GalT7. We generated a compound that both impact in vitro galactosyltransferase activity and affect GAG synthesis in cells, opening promising pharmacological applications. This molecule also represents a valuable chemical tool to explore the biological effects of GAGs.

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