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Short communication

Design, synthesis, and evaluation of a novel fluorescent probe for competitive fluorescence polarization assay to screen galectin-8 inhibitors

EDVIN PURIĆ[1]
MARINA MARINOVIĆ[1]
ZALA KOJEK[1]
BARBRO KAHL-KNUTSON[2]
HAKON LEFFLER[2]
ULF J. NILSSON[3]
MARKO ANDERLUH[1,*]
JANEZ MRAVLJAK[1,*]

[1] *Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Ljubljana, 1000 Ljubljana Slovenia*

[2] *Section MIG (Microbiology, Immunology, Glycobiology) Department of Laboratory Medicine, Lund University 22362 Lund, Sweden*

[3] *Department of Chemistry, Lund University, Box 124 221 00, Lund, Sweden*

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ABSTRACT

In the present work, we describe the design, synthesis, and evaluation of a galectin-8-binding fluorescent probe designed for a competitive fluorescence polarization (FP) assay for screening new galectin-8N inhibitors. The probe was characterized for its photophysical properties and its binding affinity for galectin-8N was determined by using FP. We evaluated the probe in a competitive FP assay with three known galectin-8N inhibitors and demonstrated its suitability for high-throughput screening.

Keywords: fluorescence polarisation assay, carbohydrates, lectins, galectin-8

INTRODUCTION

Galectin-8 is a member of the tandem-repeat galectin family that it expressed in numerous tissues. It is involved in many cellular processes and directs pathological processes in various diseases (1–4). It has a role in both innate and adaptive immune responses, where it triggers the T-cell response, regulates B-cell activation, induces the secretion of proinflammatory cytokines, and is involved in the defense against microbial infections (5, 6). Given these important functions of galectin-8, we would like to emphasize the importance of developing potent and selective galectin-8 inhibitors and the assays to screen them.

The binding affinities of galectin-8 (or galectins in general) have conveniently been determined with a competitive fluorescence polarization (FP) assay (7–10). FP is a robust technique widely used in biochemical and pharmaceutical research to study enzyme kinetics, antigen-antibody interactions, small molecule-protein interactions, protein-

* Correspondence; e-mail: marko.anderluh@ffa.uni-lj.si, janez.mravljak@ffa.uni-lj.si

protein interactions, *etc.* (11). It differentiates between the target-bound and unbound states of a fluorescently labelled ligand (fluorescent probe) in a solution without the need for further steps (like washing, *etc.*). Therefore, it enables real-time measurements in solution and is very well suited for high-throughput formats, making it an excellent method for screening compound libraries (12). The quality of the FP assay largely depends on the characteristics of the fluorescent probe used in the assay. Ideally, the probe should have a high quantum yield and photostability. High quantum yield is particularly important for probes involved in strong affinity interactions that require low probe concentrations (12). To conduct a competitive binding fluorescence polarization assay, the development of a fluorescent probe is therefore the most important step. In competitive assays, the fluorescent probe competes with a non-labelled ligand (usually an inhibitor) for binding to the protein/enzyme, and consequently, the probe should allow efficient displacement by the tested inhibitors. According to the BioAssay Research Database (BARD), the most commonly used fluorophore is fluorescein and is often used as a fluorescent label for FP assays (13).

In our work, we describe the design and characterization of a novel fluorescein-based probe specifically designed for competitive FP assay to screen novel galectin-8N inhibitors. Following the probe synthesis, the probe was characterized for its photophysical properties and its binding affinity for galectin-8N was determined by using FP. In addition, we evaluated the probe in a competitive FP assay with three known galectin-8N inhibitors to demonstrate its potential use for high-throughput screening.

EXPERIMENTAL

General

Commercially available reagents were used without any further purification. TLC (thin-layer chromatography) was performed on Merck 60 F254 silica gel plates (0.25 mm) under visualization with UV light (254 nm), ninhydrin, and phosphomolybdic acid stain. Flash column chromatography was performed on silica gel 60 with particle size 240–400 mesh. ^{1}H and ^{13}C NMR spectra were recorded at 400 MHz and 101 MHz, respectively, using a Bruker AVANCE III 400 MHz NMR spectrometer (Bruker Corporation, USA) at ambient temperature in DMSO-d_6 with tetramethylsilane (TMS) as an internal standard. Chemical shifts are reported in δ parts per million (ppm), with multiplicity, coupling constants (in Hz), and integration. High-resolution mass spectrometry (HRMS) was performed using Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Germany). Reversed-phase high-performance liquid chromatography (HPLC) analysis was performed on the Thermo Scientific Dionex UltiMate 3000 modular system (Thermo Fisher Scientific Inc., USA), where a C18 column (1.8 μm, 2.1 mm × 50 mm; Waters ACQUITY UPLC HSS C18 SB Column) was used, with a flow rate of 0.4 mL min^{-1} and sample volume of injection of 1–5 μL (mobile phase: 0.1 % trifluoroacetic acid (TFA) (*V*/*V*) in ultrapure water (solvent A) and acetonitrile (CH_3CN) (solvent B) with a following gradient: 95 % A to 5 % A in 10 min, then 95 % B for 4 min, with flow rate of 0.3 mL min^{-1} and injection volume of 5 μL, t = 40 °C). The structures were drawn with ChemDraw 20.0 (PerkinElmer), and the NMR spectra were analyzed with MestReNova v12.0.0–20080 (© 2017 Mestrelab Research S.L.). Binding curves and binding properties were analyzed with GraphPad Prism 10.2.3.

Chemistry

Synthesis of fluorescent probe **3**. – The synthesis of **1** has been described previously (14). Compound **1** (40 mg, 70.5 μmol, 1 molar equiv.), *N*-(3-azidopropyl)-3',6'-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthene]-5-carboxamide or 5-FAM-azide **2** (39 mg, 84.6 μmol, 1.2 molar equiv.), $CuSO_4 \times 5\ H_2O$ (4 mg, 14.1 μmol, 0.2 molar equiv.) and sodium ascorbate (5 mg, 28.2 μmol, 0.4 molar equiv.) were dissolved in 4 mL DMF/H_2O = 4/1 and left stirring at 40 °C overnight. The next day, the mixture was concentrated *in vacuo* and the crude material was purified with Isolera One Flash Chromatography Instrument (Biotage AB, Sweden) on reversed-phase silica gel C18 to obtain **3** as a yellow solid (30 mg; yield = 41.5 %). ^{1}H NMR (400 MHz, DMSO-d_6, δ): 9.00 (s, 1H), 8.62 (s, 1H), 8.12 (d, J = 6.9 Hz, 3H), 7.87 (d, J = 8.5 Hz, 1H), 7.77 (d, J = 2.1 Hz, 1H), 7.54 (t, J = 7.6 Hz, 2H), 7.46 (dd, J = 8.5, 2.1 Hz, 1H), 7.30 (d, J = 8.0 Hz, 1H), 6.59 (d, J = 8.9 Hz, 2H), 6.51 (d, 2H), 6.44 (d, J = 8.7 Hz, 2H), 6.09 (d, J = 5.3 Hz, 1H), 4.92 (dd, J = 37.6, 12.5 Hz, 2H), 4.71 (dd, J = 57.1, 12.2 Hz, 2H), 4.42 (t, J = 6.9 Hz, 2H), 4.22–4.10 (m, 2H), 3.99 (t, J = 6.2 Hz, 1H), 3.78 (s, 3H), 3.62–3.48 (m, 2H), 3.42–3.31 (m, 3H), 2.10 (t, 2H) ppm. ^{13}C NMR (101 MHz, DMSO-d_6, δ): 185.43, 168.16, 167.57, 164.79, 159.68, 159.60, 154.67, 151.81, 136.12, 135.46, 134.74, 134.71, 132.84, 132.38, 131.31, 131.28, 130.56, 129.49, 129.23, 129.14, 129.10, 128.54, 128.00, 126.59, 126.41, 125.29, 124.21, 124.19, 123.28, 112.66, 109.06, 102.27, 86.03, 74.59, 72.37, 64.98, 60.01, 57.93, 47.35, 39.52, 36.69, 30.40, 29.67, 23.04, 20.74, 19.18, 14.07, 13.46, 7.59 ppm. HRMS-ESI (m/z): $[M + H]^+$ calcd. for $C_{49}H_{43}Cl_2O_{13}N_6S$: 1025.19804; found, 1025.19581. HPLC purity: 99.59 %. Yellow amorphous solid.

Biological evaluation

Characterization of fluorescent probe **3**. – Excitation (absorbance) and emission spectra were measured with Spark® multimode microplate reader, Tecan Group Ltd., Switzerland. 12 different concentrations of probe **3** were prepared and excitation (absorbance) spectra were measured. Excitation and emission peaks were measured as depicted in the Fig. 2.

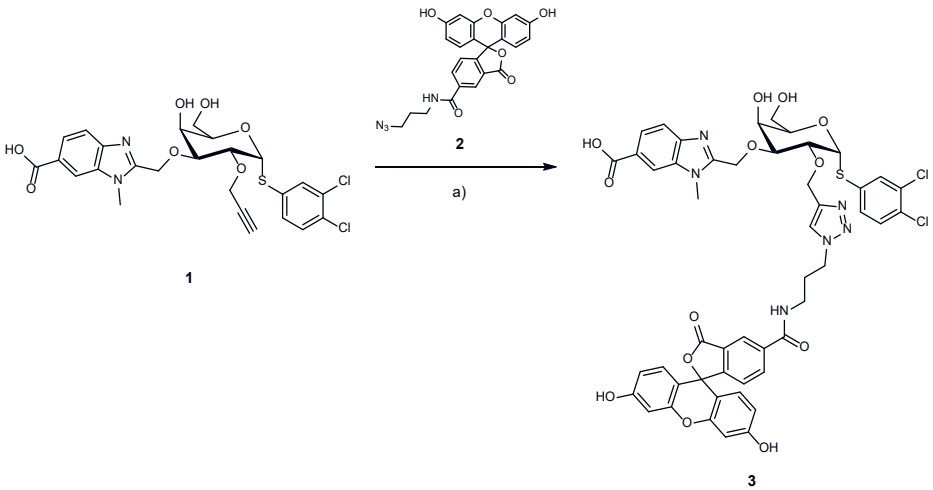
Fluorescence polarization experiments. – Recombinant human galectins-1, -3, and -8N were expressed and purified as previously described (7–9). Fluorescence polarization assays were conducted using a PHERAstar FS plate reader equipped with PHERAstar Mars version 2.10 R3 software (BMG, Germany), as well as a Spark® multimode microplate reader. The fluorescence anisotropy of fluorescein-labeled probe **3** was measured with an excitation wavelength of 495 nm and an emission wavelength of 535 nm. A 20 mmol L^{-1} stock solution of probe **3** was prepared in pure DMSO and subsequently diluted in 1 % DMSO in PBS buffer (pH = 7.4) to obtain 8–15 different concentrations. Galectins were titrated against a fixed concentration of probe **3** (0.1 μmol L^{-1}). Additionally, a competitive fluorescence polarization assay was performed to determine the binding affinities of three known galectin-8N inhibitors. In this assay, a fixed concentration of galectin-8N (0.4 μmol L^{-1}) and probe **3** (0.1 μmol L^{-1}) were used. Compounds **1**, **4**, and **5** (structures shown in Fig. 4) were initially dissolved in pure DMSO at 20 mmol L^{-1} and then diluted in 5 % DMSO in PBS to obtain seven different concentrations. Each concentration was tested in triplicate using serial dilutions starting at 30 μmol L^{-1}. The dissociation constant (K_d) and standard error of the mean (SEM) were calculated from seven triplicate measurements, covering an inhibition range of 10–90 %. Additionally, Z and Z′ parameters were determined to assess the method's suitability for high-throughput screening (HTS) applications.

RESULTS AND DISCUSSION

*Synthesis and characterization of fluorescent probe **3***

An important feature of the fluorescent probe for the fluorescence polarization assay is its affinity for various galectins (15, 16). Based on the latest studies (14–18) and the availability of structural data, we designed a fluorescent probe **3**, starting from the known galectin-8N inhibitor **1**, which reached the submicromolar/high nanomolar affinity for galectin-8N (14). Positions 1 and 3 of D-galactose were previously optimized and since, according to the availability of structural data (PDB ID: 7AEN) 4-OH and 6-OH of D-galactose must remain unsubstituted, so position 2 remained suitable for further derivatization. The highly versatile propargyl group, present in the structure of **1** opens new synthetic pathways for further modifications, such as azide-alkyne cycloaddition reactions. As shown in Scheme 1, compound **1** was, therefore, used in a copper-catalyzed azide-alkyne click reaction with the corresponding 5-FAM azide **2** to obtain probe **3**.

In our previous works, a tetrasaccharide probe **6** (containing LNnT, Fig. 1) was used to determine the K_d values of galectin-8N inhibitors (9, 10). The above-mentioned probe **6** contains a fluorescein moiety, which was the main reason why we used fluorescein in the synthesis of the new probe **3**. However, the synthesis of probe **6** starts from lacto-*N*-neotetraose (LNnT) with a 2-azidoethyl linker, which is converted to free amine in a Staudinger reaction and later coupled with 5-carboxyfluorescein (9, 10). The LNnT tetrasaccharide offers potent binding to galectin-8N, but is laborious to synthesize, as its synthesis requires 15 steps and limits the wide applicability of the probe (19). Comparatively, the synthesis of probe **3** started from the known galectin-8N inhibitor **1**, which was reacted with 5-FAM-azide **2** in a simple copper-catalyzed azide-alkyne cycloaddition (CuAAC). Since the synthesis of the precursor **1** is much easier (7 steps *vs.* 15 steps for LNnT) than the synthesis of LNnT, it is possible to prepare probe **3** in larger quantities if needed.



Scheme 1. Reagents and conditions: a) $CuSO_4 \cdot 5\,H_2O$, sodium ascorbate, DMF/H_2O = 4/1, 40 °C, overnight.

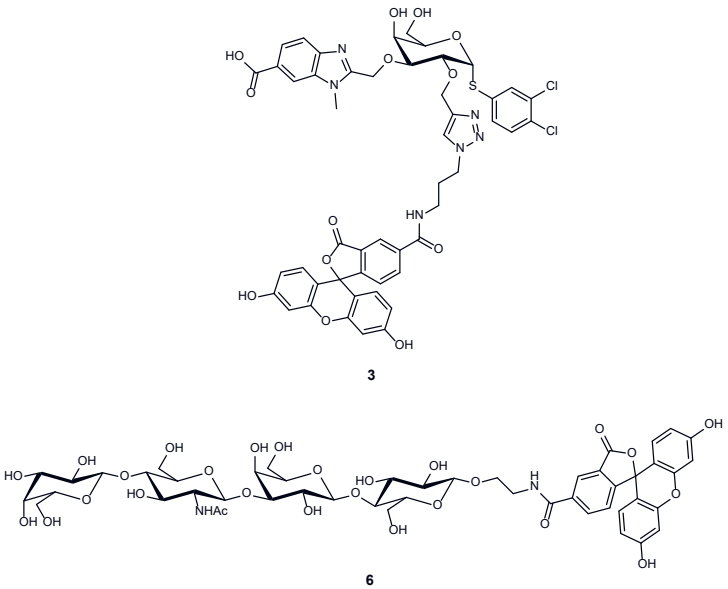


Fig. 1. Structures of fluorescent probes **3** and **6** (9, 10) used in fluorescence polarization assays.

The fluorescent probe **3** was further characterized using UV-VIS spectroscopy (Fig. 2a). The absorption spectra were recorded in PBS buffer at 20 °C and showed that the maximum of the main absorption peak of **3** is at λ_{ex} = 500 nm. In Fig. 2b, the fluorescence emission spectra of **3** show a maximum emission peak at λ_{em} = 532 nm.

*Binding studies with fluorescent probe **3***

The binding properties of **3** were evaluated on galectin-1, -3, and -8N using a fluorescence polarization assay (7, 8). As expected, it exhibits the highest affinity for galectin-8N (0.94 ± 0.16 μmol L^{-1}) as it contains the glycan part designed for targeting galectin-8N. It shows a good selectivity over galectin-1, but not towards galectin-3, as expected based on

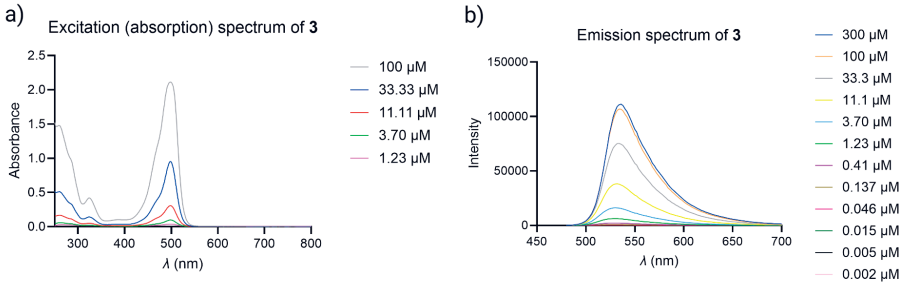


Fig. 2. a) Excitation (absorbance) and b) emission spectra of **3** in PBS buffer (pH = 7.4).

similarities with the galectin-8N binding site (Table I and Fig. 3). This fact however is not detrimental, as it allows the probe to be used for competitive binding studies for both galectin-3 and 8N.

*Table I. Binding properties of **3** against galectin-8N, galectin-3 and galectin-1*

	Gal-8N	Gal-3	Gal-1
K_d (μmol L^{-1})	0.94 ± 0.16	5.24 ± 0.23	72.7 ± 5.1
A_{max} (mA)[a]	210	226	226
A_0 (mA)[b]	35.9	32.9	33.2

[a] A_{max} is the maximum anisotropy value for galectin-probe complex, [b] A_0 is the anisotropy of the free probe.

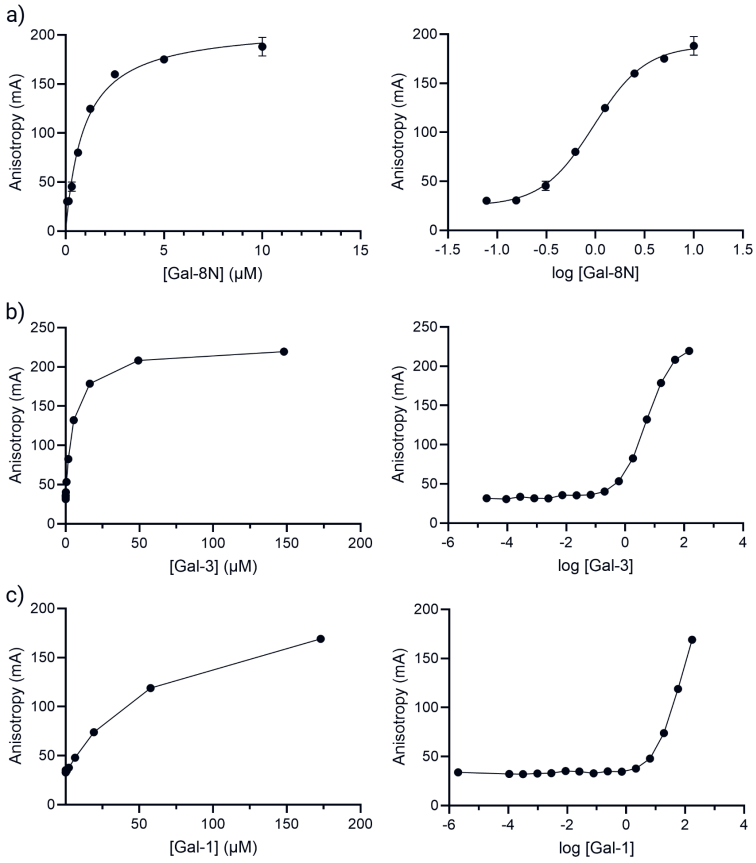


Fig. 3. Binding of: a) galectin-8N; b) galectin-3; c) galectin-1 to fluorescent probe **3**. A fixed concentration of probe (0.1 μmol L^{-1}) was mixed with a range of concentrations of galectins (x-axis), and fluorescence anisotropy was measured (y-axis). Standard devation (SD) values are plotted as whiskers, but are visible only for some experimental points due to their low values.

Another intriguing aspect of probe **3** is the fluorescein conjugation point and its influence on the behavior of the probe. This is the first probe for galectins in which fluorescein was conjugated to position 2 of the D-galactoside, in contrast to the oligosaccharide probes of Carlsson *et al*. in which fluorescein was attached to C1 of the reducing sugar in natural glycans, all pointing into subsite E of the galectin-8N (6, 9). Position 2 was chosen as the attachment point because it points toward the solvent, as indicated by our recent crystal structure of **1** in complex with Gal-8N (PDB ID: 9FYJ) (14). However, if the fluorescein moiety pointed toward the solvent, the fluorescein moiety would be expected to rotate more freely and give a lower A_{max}. Yet, the measured A_{max} (Table I) shows that this is not the case. This could be due to the fluorescein moiety binding to protein subsite B, which directly hinders the rotation of the probe and results in more polarized light and a higher A_{max} readout. The assumption was corroborated by docking probe **3** to the crystal structure of **1** in complex with Gal-8N (PDB ID: 9FYJ), which shows a similar binding pose for **3** compared to **1**, with the fluorescein moiety leaning towards the solvent and coming into contact with Arg59 in the protein subsite B (please see Fig. S4 in Supplementary data). The influence of the additional contact is probably negligible in terms of affinity, as the major part of **3** points towards the solvent. Interestingly, this also appears to be the case for galectins 3 and 1. Although this does not affect the usefulness of the probe in high-throughput screening assays, it does increase the assay dynamic range and the ultimate sensitivity of the assay.

Probe **3** was used in a competitive fluorescence polarization assay. The binding affinities of three potent galectin-8N inhibitors were determined and compared with the affinities determined in a competitive fluorescence polarization assay by using probe **6**. The affinities of known galectin-8N inhibitors D-lactose, **1**, **4,** and **5** (Fig. 4) are very similar to those determined in the FP assay with probe **6** (comparison of K_d values is shown in Table II), suggesting that a newly synthesized probe **3** can be used alternatively in the screening of galectin-8N inhibitors with the FP assay.

*Table II. Gal-8N K_d values (μmol L^{-1}) of D-lactose, **1**, **4**, and **5** using two fluorescent probes **3** and **6**[a, b]*

Gal-8N inhibitor	K_d with probe **3**	K_d with probe **6**
D-lactose	54 ± 9	80 ± 10
1	0.82 ± 0.09	0.80 ± 0.09
4	2.2 ± 0.1	1.8 ± 0.0
5	0.48 ± 0.07	0.50 ± 0.03

[a] Results represent the mean ± SEM of $n = 4$ to 8.[b] K_d was determined by competitive fluorescence polarization assay.

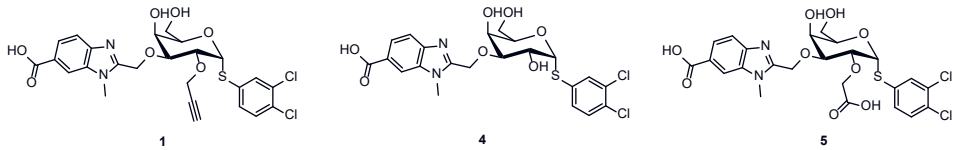


Fig. 4. A selection of three known galectin-8N inhibitors (14, 16), which were used in a competitive fluorescence polarization assay.

Furthermore, the Z and Z′ factors were calculated (see Table III, and Tables S1 and S2) to validate the fluorescence polarization assay for high-throughput screening (HTS). The Z-factor is a simple and useful tool for assay quality evaluation and defines a parameter for hit identification ability for any given assay under specified screening conditions (20). Z values can vary from $-\infty < Z \leq 1$ but are only relevant in the range of $-1 < Z < 1$, since at $Z = -1$ the lower detection limit of the assay system is already reached. In contrast, the Z′-factor is a parameter for determining the assay quality itself (without the use of compounds/agonists/antagonists or inhibitors) and is therefore not limited to HTS assays. In any case with large data sets, Z values are always lower or equal Z′. Consequently, low Z′ values reflect the need to optimize assay conditions (20). Since the highest galectin-8N inhibitor concentration used in the FP assay is the most critical (due to possible low solubility/aggregation), only the Z-factor values for the highest galectin-8N inhibitor concentration tested are shown in Table III, while the Z-factor values for all concentrations are shown in Tables S1 and S2. According to the calculations (see Table III, and Tables S1 and S2), the Z-factor values for both fluorescent probes **3** and **6** are greater than 0.5, indicating that the fluorescence polarization assay is a suitable assay for screening galectin-8N inhibitors in a high-throughput fashion. Moreover, the Z′-factor values are 0.845 for probe **3** and 0.863 for probe **6**, indicating that the assay conditions and controls were properly selected and optimized.

*Table III. Z and Z′-factor calculations for fluorescent probes **3** and **6**. These are Z-factor values for the highest inhibitor concentrations*

Gal-8N inhibitor	Z (for probe **3**)	Z (for probe **6**)	Z′ (for probe **3**)	Z′ (for probe **6**)
1	0.715	0.687		
4	0.834	0.604	0.845	0.863
5	0.550	0.504		

CONCLUSIONS

In summary, we have designed a galectin-8-binding fluorescent probe **3** for a competitive fluorescence polarization assay to screen novel galectin-8N inhibitors. The fluorescent probe **3** exhibited a high nanomolar affinity for galectin-8N and showed good selectivity against galectin-1, but not against galectin-3 as expected. In addition, fluorescent probe **3** was used in a competitive FP assay, in which the binding affinities of three known galectin-8N inhibitors were determined. The binding affinities were very similar to those obtained in the FP assay with fluorescent probe **6**. Therefore, probe **3** can alternatively be used in a competitive FP assay to screen new galectin-8N inhibitors. Moreover, the synthesis of probe **3** is simpler and proceeds with a higher yield compared to the synthesis of probe **6**. Based on the calculations of the Z- and Z′-factor, we have shown that fluorescent probes **3** and **6** are suitable for high-throughput screening of galectin-8N inhibitors.

Abbreviations, acronyms, symbols. – BARD – BioAssay Research Database, CuAAC – copper-catalyzed azide-alkyne cycloaddition, DMF – dimethyl formamide, DMSO – dimethyl sulfoxide, ESI – electrospray ionization, FP – fluorescence polarization, HPLC – high-performance liquid chromatography, HRMS – high-resolution mass spectrometry, HTS – high throughput screening, NMR – nuclear

magnetic resonance, PBS – phosphate buffer saline, SEM – standard error mean, TFA – trifluoroacetic acid, TLC – thin-layer chromatography, TMS – tetramethylsilane.

Supplementary data. – In the supplementary file, NMR (^{1}H and ^{13}C) and HPLC data of probe **3** are given. In addition, detailed Z and Z'-factor calculations for probes **3** and **6** are presented in Tables S1 and S2.

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Conflicts of interest. – U.J.N. and H.L. are shareholders in Galecto Biotech Inc., a company developing galectin inhibitors.

Authors contributions. – Conceptualization, M.A., E.P. and J.M.; data curation, E.P., Z.K., J.M. and B.K.K., investigation, E.P. and J.M.; formal analysis, E.P.; methodology, E.P., M.M., Z.K., J.M. and B.K.K.; validation, E.P., M.M., J.M. and B.K.K.; visualization, J.M., M.M., U.J.N. and B.K.K.; software, E.P., Z.K. and B.K.K.; writing original draft, E.P., Z.K., M.A. and J.M.; writing – review and editing, M.A., J.M., B.K.K. and U.J.N.; supervision, M.A., J.M. and U.J.N.; project administrator, M.A. All authors have read and agreed to the published version of the manuscript.

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Supporting information for the article

Design, synthesis, and evaluation of a novel fluorescent probe for competitive fluorescence polarization assay to screen galectin-8 inhibitors

Edvin Purić[1], Marina Marinović[1], Zala Kojek[1], Barbro Kahl-Knutson[2], Hakon Leffler[2], Ulf J. Nilsson[3], Marko Anderluh[1,*], Janez Mravljak[1,*]

[1] *Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Ljubljana, 1000 Ljubljana, Slovenia*

[2] *Section MIG (Microbiology, Immunology, Glycobiology), Department of Laboratory Medicine, Lund University, 22362 Lund, Sweden*

[3] *Department of Chemistry, Lund University, Box 124, SE-221 00, Lund, Sweden*

[*] Corresponding authors: marko.anderluh@ffa.uni-lj.si, janez.mravljak@ffa.uni-lj.si

^{1}H, ^{13}C NMR and HPLC chromatogram of **3**

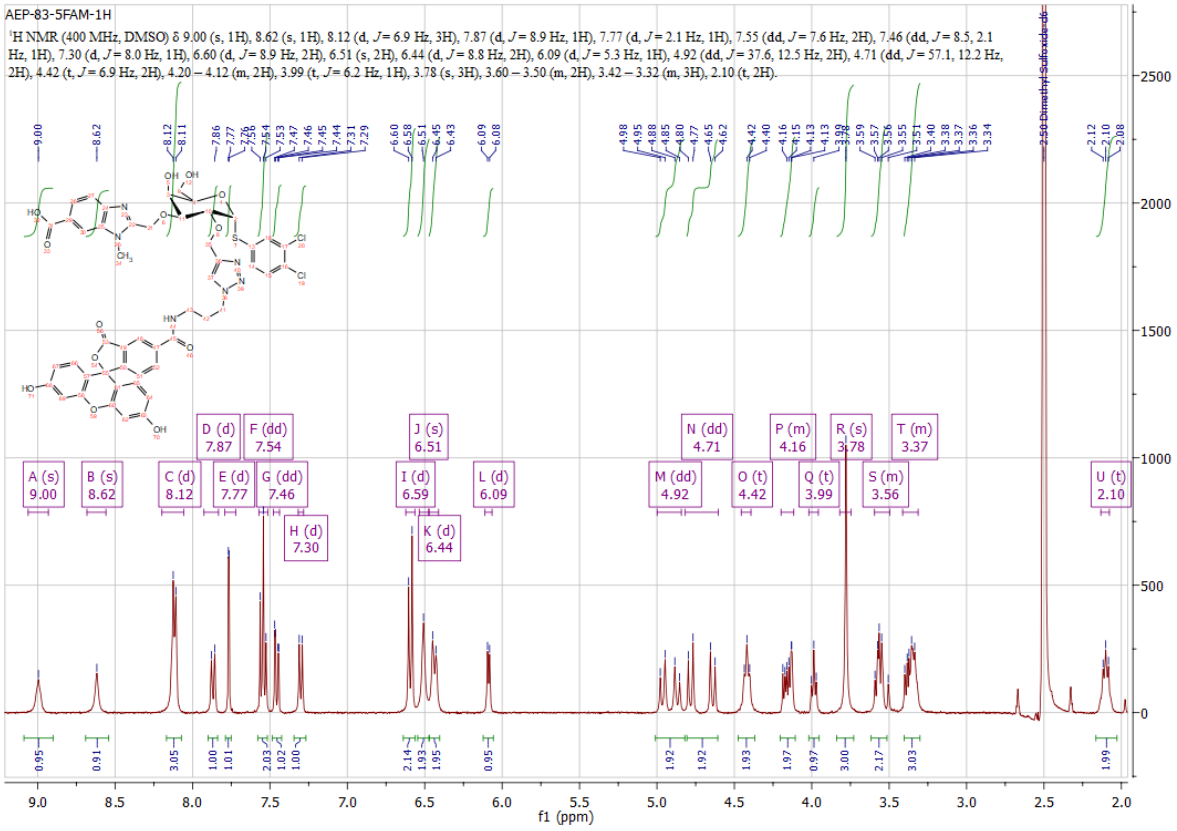


Fig. S1. ^{1}H NMR (400 MHz, DMSO-d_6) of **3**.

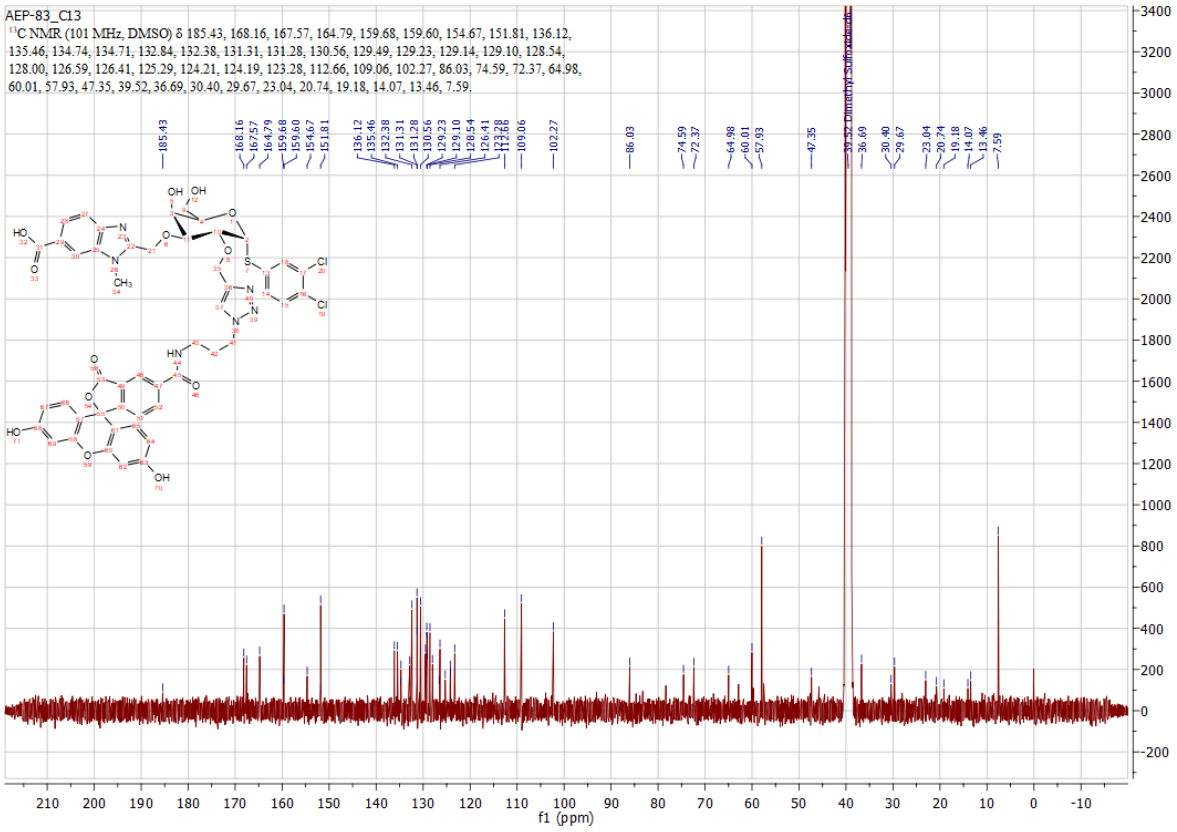
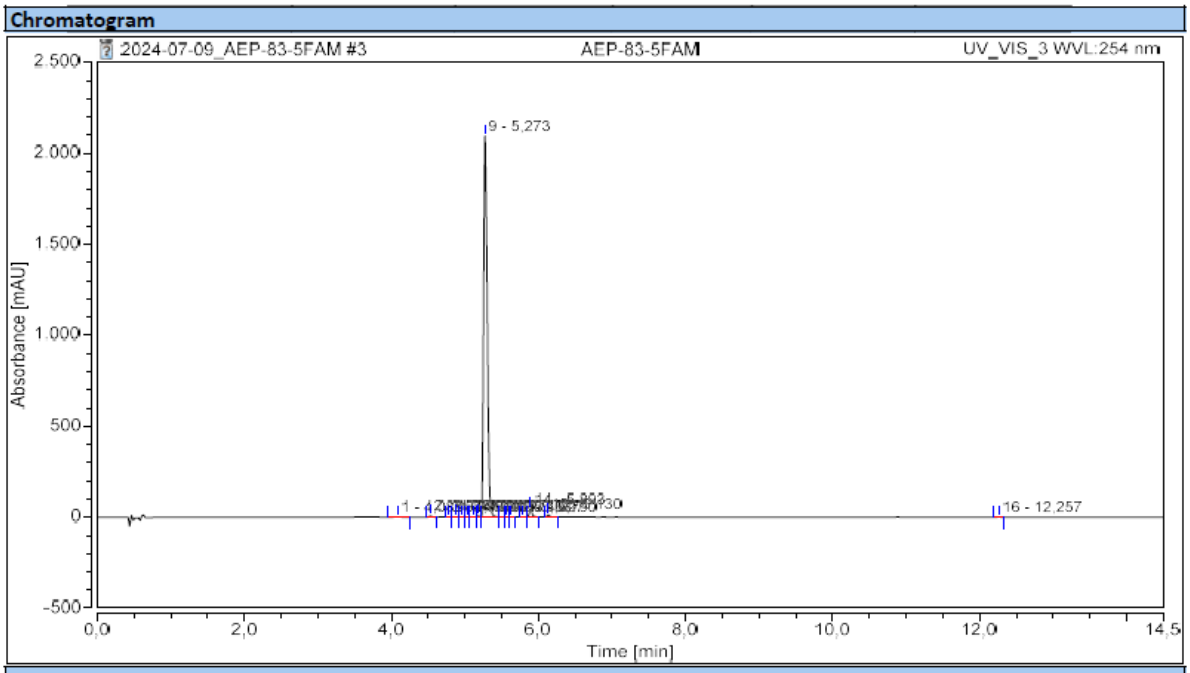


Fig. S2. ^{13}C NMR (101 MHz, DMSO-d_6) of **3**.



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		4,083	0,122	1,850	0,10	0,08	n.a.
2		4,530	0,288	7,471	0,24	0,34	n.a.
3		4,767	0,081	2,083	0,07	0,10	n.a.
4		4,867	0,120	3,147	0,10	0,14	n.a.
5		4,947	0,069	1,605	0,06	0,07	n.a.
6		5,040	0,035	0,738	0,03	0,03	n.a.
7		5,117	0,112	2,542	0,09	0,12	n.a.
8		5,187	0,257	6,715	0,21	0,31	n.a.
9		5,273	118,450	2093,580	97,07	95,98	n.a.
10		5,467	0,071	1,540	0,06	0,07	n.a.
11		5,567	0,038	0,794	0,03	0,04	n.a.
12		5,627	0,069	1,641	0,06	0,08	n.a.
13		5,790	0,034	0,821	0,03	0,04	n.a.
14		5,893	1,722	42,990	1,41	1,97	n.a.
15		6,130	0,521	13,110	0,43	0,60	n.a.
16		12,257	0,032	0,624	0,03	0,03	n.a.
Total:			**122,020**	**2181,252**	**100,00**	**100,00**	

Fig. S3. HPLC-UV spectrum of **3**.

Biological evaluation

Characterization of fluorescent probe **3**. – Excitation (absorbance) and emission spectra were measured with Spark® multimode microplate reader, Tecan Group Ltd., Männedorf, Switzerland. 12 different concentrations of probe **3** were prepared and excitation (absorbance) spectra were measured. Excitation and emission peaks were measured as depicted in the Fig. 2.

Fluorescence polarization experiments. – Human galectins-1, -3, and -8N were expressed and purified as described previously (1–3). Fluorescence polarization assays were performed using a PHERAstar FS plate reader with PHERAstar Mars version 2.10 R3 software (BMG,

Offenburg, Germany) and a Spark® multimode microplate reader, where a fluorescence anisotropy of the fluorescein-labelled probe **3** was measured by excitation at 495 nm and emission at 535 nm. Briefly, a 20.0 mM stock solution of **3** was prepared in pure DMSO and later diluted in 1 % DMSO in PBS solution (pH = 7.4) to 8–15 different concentrations. The corresponding galectin was titrated against a fixed concentration of **3** (0.100 μM). In addition, the fluorescent probe **3** was used in the competitive fluorescence polarization assay, where the binding affinities of three selected known galectin-8N inhibitors were determined. A fixed concentration of galectin-8N (0.400 μM) and probe **3** (0.100 μM) were used. Compounds **1**, **4** and **5** (structures shown in Figure 4 in the main text) were dissolved in pure DMSO at a concentration of 20 mM and later diluted in 5 % DMSO in PBS solution to 7 different concentrations. Each concentration was tested in triplicate by serial dilutions starting from 30.0 μM. The average values of K_d and SEM were calculated from 7 triplicate measurements, showing 10–90 % inhibition. Z and Z′ parameters were calculated to validate the suitability of the method for HTS purposes.

*Table S1. Calculated Z′ and Z values for fluorescent probe **3***

Galectin-8N inhibitor	Z	Concentration (μM)	Z'
1	0.715	30.0	0.845
	0.720	15.0	
	0.569	7.50	
	0.731	3.75	
	0.692	1.88	
	0.838	0.938	
	0.843	0.469	
4	0.834	30.0	
	0.669	15.0	
	0.641	7.50	
	0.786	3.75	
	0.765	1.88	
	0.821	0.938	
	0.844	0.469	
5	0.550	30.0	
	0.683	15.0	
	0.823	7.50	
	0.821	3.75	
	0.709	1.88	

	0.836	0.938	
	0.843	0.469	

*Table S2. Calculated Z′ and Z values for tetrasaccharide probe **6***

Galectin-8N inhibitor	Z	Concentration (μM)	Z'
1	0.687	10.0	0.863
	0.749	5.00	
	0.660	2.50	
	0.650	1.25	
	0.854	0.625	
	0.824	0.313	
4	0.504	20.0	
	0.573	10	
	0.732	5.00	
	0.731	2.50	
	0.667	1.25	
	0.820	0.625	
	0.602	0.313	
5	0.604	30.0	
	0.577	10.0	
	0.837	3.33	
	0.848	1.11	
	0.849	0.370	

Docking studies

Ligand preparation. – The structure of the probe **3** was built with ChemDraw Professional 23.1.1 (Revvity Signals Software, Inc.) and their geometries optimized with Chem3D 23.1.1 (Revvity Signals Software, Inc.) using MM2 force field until a minimum 0.100 Root Mean Square (RMS) gradient was reached. The optimized structure was refined with GAMESS interface using the semi-empirical AM1 method, QA optimization algorithm and Gasteiger–Hückel charges for all atoms for 100 steps. FRED requires a set of input conformers for each ligand, which were generated with OMEGA (OMEGA version 5.0.0.3. OpenEye Scientific Software, Santa Fe, NM. http://www.eyesopen.com), with maximum number of conformations set to 200 (4,5). All the other options were left as default values.

Receptor preparation and docking protocol. – Crystal structure with co-crystalized Gal-8N inhibitor **1** in complex with Gal-8N (PDB code: 9FYJ) was taken as the starting point (6). Receptor was prepared as a large grid box that surrounded the binding site of **1** with the volume of 47572 $Å^3$, dimensions: 40.19 Å × 29.35 Å × 40.34 Å, and outer contour of 21086 $Å^2$ using Make Receptor 4.3.0.3. with "Balanced Site Shape Potential" and the following constraints: ARG45 HB Acc, ARG59 HB Acc, HIS65 HB Don, ASN79 HB Acc, GLU89 HB Don (7).

The docking software FRED (OEDocking version 4.1.1.0 OpenEye Scientific Software, Santa Fe, NM. http://www.eyesopen.com) was used for docking studies with the default settings,

and number of poses, which was set to 50 (8, 9). The binding pose with the highest rank of the docked probe **3** is presented in the Figure S4. The graphical representation of the calculated binding pose was obtained using Vida (version 5.0.1.0, OpenEye Scientific Software, Santa Fe, NM. http://www.eyesopen.com).

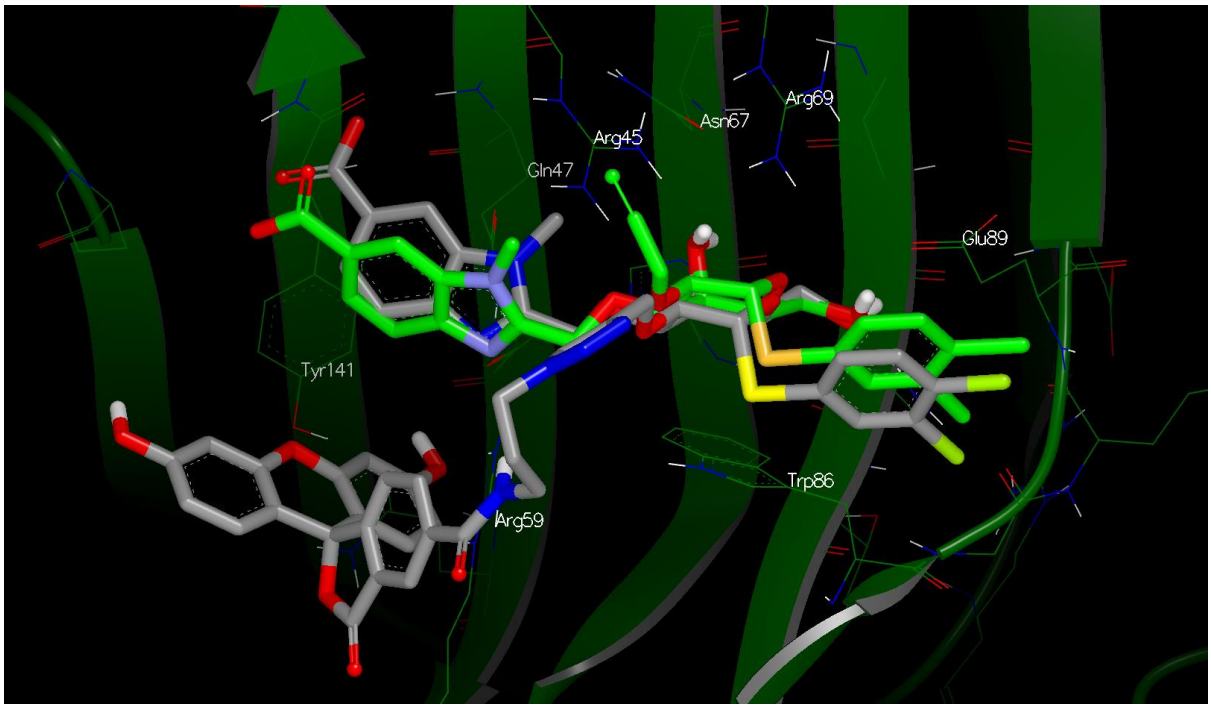


Fig. S4. The fluorescent probe **3** (shown in sticks, carbons in grey) docked into the crystal structure of inhibitor **1** (shown in sticks, carbons in green) in complex with Gal-8N (protein depicted as green ribbons with selected amino acid residues in lines, PDB code: 9FYJ) (6). The docked pose shows a similar binding pose for **3** compared to **1**, with the fluorescein moiety leaning towards solvent and making contact (hydrogen bond) with the guanidine of Arg59.

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