ACES Asian Chemical Editorial Society

doi.org/10.1002/asia.202200228

CHEMISTRY
AN ASIAN JOURNAL
Research Article

www.chemasianj.org

Synthesis of Novel Glycolipid Mimetics of Heparan Sulfate and Their Application in Colorectal Cancer Treatment in a Mouse Model

Sam Spijkers-Shaw[+],[a] Katrin Campbell[+],[b] Nicholas J. Shields,[b, c] John H. Miller,[d] Phillip M. Rendle,[a] Wanting Jiao,[a] Sarah L. Young,[b, c] and Olga V. Zubkova*[a]

Dedicated to Professor Peter C. Tyler on the occasion of his 65th birthday.

Abstract: Heparan sulfate (HS) is a highly sulfated natural carbohydrate that plays crucial roles in cancer, inflammation, and angiogenesis. Heparanase (HPSE) is the sole HS degrading endoglycosidase that cleaves HS at structure-dependent sites along the polysaccharide chain. Overexpression of HPSE by cancer cells correlates with increased tumor size and enhanced metastasis. Previously we have shown that a tetramer HS mimetic is a potent HPSE inhibitor displaying remarkable anticancer activity *in vivo*. Building on that work, we report the synthesis and testing of a novel library of single entity trimer glycolipid mimetics that effectively inhibit HPSE at low nanomolar concentrations. A lipophilic arm was introduced to assess whether an improvement of pharmacokinetics and plasma residence time would offset the reduction in charge and multivalency. Preclinical tests in a mouse syngeneic model showed effective tumor growth inhibition by the tetramer but not the trimer glycomimetic.

Introduction

Heparan sulfate (HS) and heparin are linear glycosaminoglycan (GAG) polysaccharides that are naturally produced by the human body.[1] HS is ubiquitously found at the cell surface whereas heparin is produced and localised in mast cells.[2] HS is predominantly found in the extracellular matrix (ECM) as a proteoglycan[3] and has a multitude of roles at a cellular level, including sequestering proteins in the extracellular matrix,[4] endocytosis,[5] and involvement in cellular signalling,[6] among many other functions.[7] Heparin is not as widely expressed and plays a limited role in homeostasis. Heparin from porcine intestine is approved for clinical use in medical settings as an anticoagulant.[8] Given that heparin has clinical approval and is available commercially, it has been investigated as a potential therapeutic for wider applications beyond anticoagulation, including the efficient treatment of viral infections.[9]

HS-protein interactions have garnered interest as the target of potential therapeutics because of their implication in a wide range of pathologies.[10] For example, HS is directly implicated in stimulating angiogenesis[11] and regulating cell proliferation.[12] However, HS and heparin are promiscuous in their protein interactions due to their sulfated nature and large size.[13] As such, our understanding of these interactions is limited, particularly as the structure of HS is not conserved, varying in sulfation and sugar composition both along the chain and between cells.[14] Recent attempts to profile the HS interactome show the far-reaching nature and complexity of HS-protein interactions.[15]

An example of a HS-interacting protein of interest is heparanase (HPSE), a mammalian β-endoglucuronidase that degrades HS into shorter length oligosaccharides.[16] HPSE expression is upregulated in nearly all cancer types examined[17] and its upregulation is concretely linked to angiogenesis,[18] metastasis[19] and poor patient prognosis.[20] Furthermore, HPSE has been found to regulate autophagy,[21] is linked to chemoresistance, and influences macrophage phenotype and recruitment to the site of a tumor.[22]

Enzymatic cleavage of HS by HPSE at the cell-surface is a main mechanism of its pro-tumorigenic and metastatic effect. The cleavage of HS releases cell signalling molecules and smaller active fragments of HS, whilst simultaneously facilitating tumor cell-motility through the partial dissolution of the basement membrane and ECM. In addition to its interactions and effects, there is only a single gene for HPSE (HPSE-1), therefore no redundant pathways exist to bypass its inhibition.[23] A second homologous gene, HPSE-2, is not enzymatically active, inhibits HPSE-1, and potentially acts as a tumor suppressor. All references to heparanse or HPSE in this paper indicate HPSE-1.

[a] S. Spijkers-Shaw,[+] Dr. P. M. Rendle, Dr. W. Jiao, Dr. O. V. Zubkova
Ferrier Research Institute
Victoria University of Wellington
6140 Wellington (New Zealand)
E-mail: olga.zubkova@vuw.ac.nz

[b] Dr. K. Campbell,[+] Dr. N. J. Shields, Prof. S. L. Young
Department of Pathology, Dunedin School of Medicine
University of Otago
9054 Dunedin (New Zealand)

[c] Dr. N. J. Shields, Prof. S. L. Young
School of Medical Sciences, Faculty of Medicine and Health
The University of Sydney
2006 Sydney (Australia)

[d] Prof. J. H. Miller
School of Biological Sciences
Victoria University of Wellington
Kelburn, 6140 Wellington (New Zealand)

[+] These authors contributed equally.

Supporting information for this article is available on the WWW under https://doi.org/10.1002/asia.202200228

Everything outlined above makes HPSE an attractive therapeutic target. As such, increasingly robust *in silico* drug design methods have been applied to HPSE since the elucidation of the enzymatic crystal structure.[24] However, the *in silico*-designed compounds developed so far have yet to reach similar inhibitory efficacy as carbohydrate-based HS mimetics.[25] Carbohydrate-based compounds targeting HPSE have previously used heparin or other naturally-sourced polysaccharides as starting materials.

Heparin is a HPSE inhibitor itself, albeit one with serious side-effects due to its potent anticoagulant action. Therefore research that focuses on modifying heparin to target HPSE attempts to reduce the anti-coagulative activity without reducing HPSE inhibition. However, using natural sources leads to a mixture of compounds and complicates establishing the exact mechanism of action or optimising the action on the target enzyme. PI-88, M402, and SST0001 are examples of modified polysaccharide HPSE inhibitors that have all reached clinical trials[26] (Figure 1). The only single-entity HPSE inhibitor to reach clinical trials is PG545 (pixatimod)[27] (Figure 1); however, it retains some anti-coagulant effects.

Aiming to address the need for an effective and affordable single-entity HPSE inhibitor devoid of anticoagulant side-effects, we have previously developed a dendrimer-based library of compounds that shows promising results *in vitro* and *in vivo*.[28] In the process, we have identified a lead compound, Tet-29[29] (Figure 1). The present work builds upon that library of compounds by investigating a simplification of the structure that could allow for future derivatisation, testing of this new library in comparison with Tet-29 *in vitro*, and tests in *in vivo* preclinical mouse models of cancer.

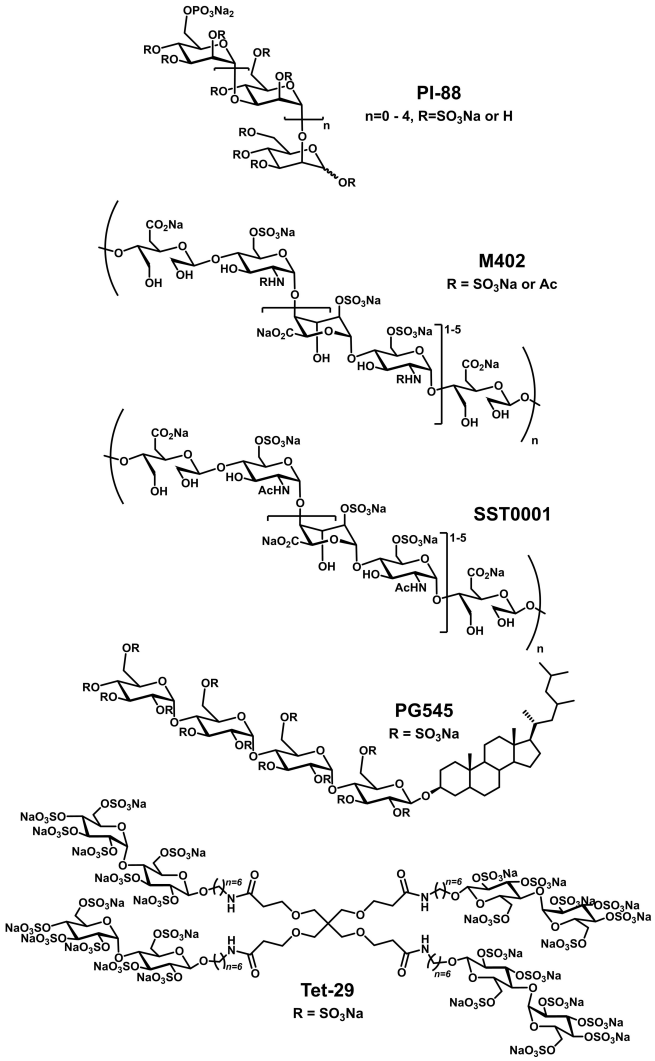


Figure 1. HS- and heparin-based mimetic heparanase inhibitors.

Results and Discussion

Synthesis of novel HS mimetics

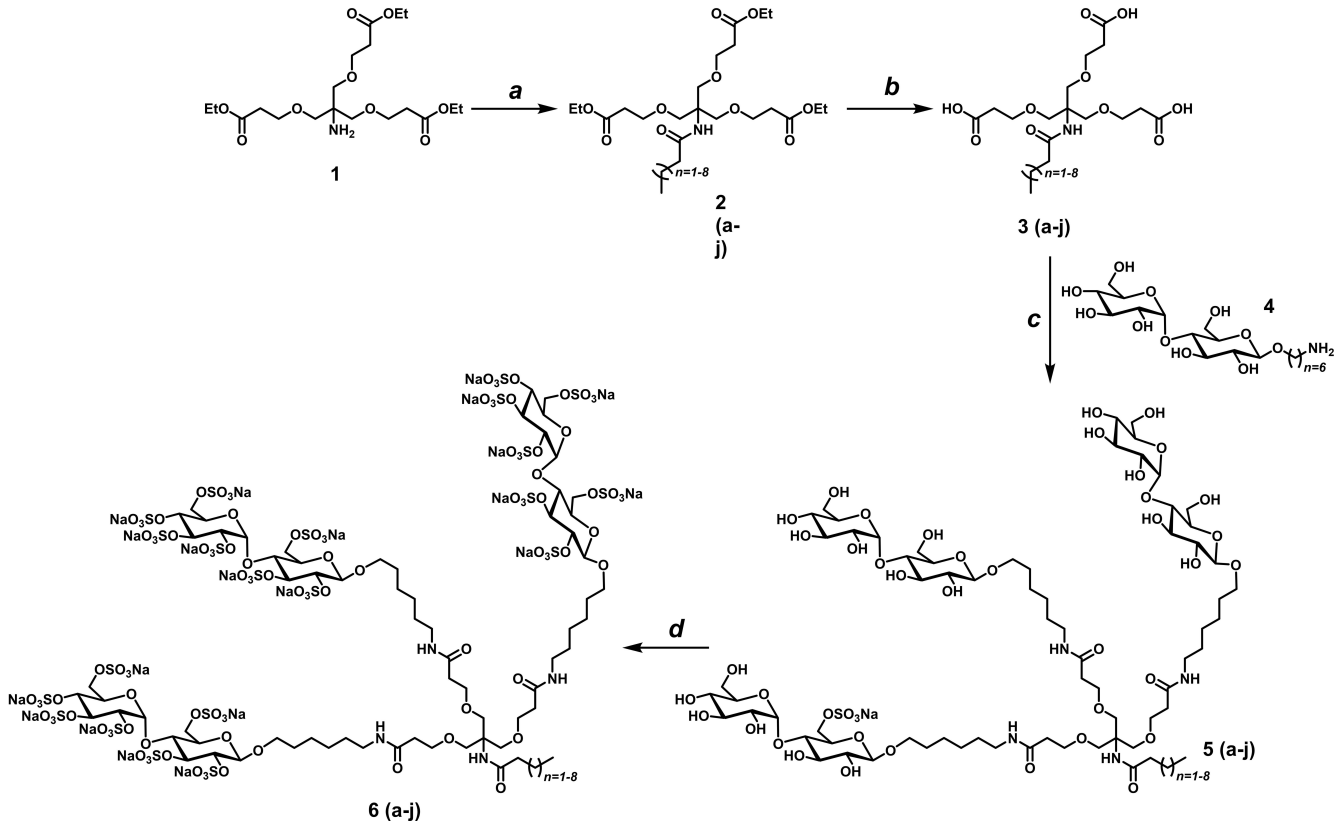
The synthesis of a set of novel trimer glycolipid HS mimetics (Scheme 1) is based upon the preparaion of Tet-29 (Figure 1), the predominant point of difference being the core scaffold. The aim of the synthesis was to simplify the compounds and make steps towards improving bioavailability. If the activities of the new glycolipids are comparable to Tet-29, then the reduction in dendritic arms from four to three provides an attachment point for potential further modification and optimisation. The synthesis starts with the production of the well-reported ester-capped core **1** (Suppl. Info.). Amide coupling to the core of fatty acids with chain lengths ranging from 4 to 13 carbons affords the group of compounds **2**(**a**–**j**). Ester groups are then removed by hydrolysis yielding carboxylic acids **3**(**a**–**j**). Previously, the synthesis of Tet-29 required *N*-hydroxysuccinimde (NHS) activation at this stage before maltose-amine (**4**) could be coupled to the core; however, a direct coupling has since been developed eschewing the need for NHS activation and reducing the number of synthetic steps.

The coupling between **3**(**a**–**j**) and **4**, giving non-sulfated glycolipids **5**(**a**–**j**), is achieved with PyBOP, which was chosen to reduce unwanted ester formation, in respectable yields.

Non-sulfated compounds **5**(**a**–**j**) are subjected to persulfation with $SO_3{\cdot}NMe_3$ to give the fully sulfated trimer glycolipids **6**(**a**–**j**), that undergo ion exchange to afford the sodium salt form. The compounds obtained were characterised via one-dimensional and two-dimensional NMR, along with high resolution mass spectrometry (Suppl. Info.).

In vitro assay: HPSE inhibition

Determination of the HPSE inhibiting efficacy of synthesised compounds was achieved with a previously reported fondaparinux cleavage assay.[30] The IC_{50} for representative compounds of the library of novel trimer glycolipids were determined and all were shown to be effective inhibitors of HPSE with IC_{50} values in the low nanomolar range (71–230 nM) (Figure 2, A). This compares well with the IC_{50} of PG545, which was measured



Scheme 1. Synthesis of novel glycolipids (a) HATU, $CH_3(CH_2)_nCH_2COOH$, DIPEA, DMF, RT; (b) NaOH, MeOH, H_2O, RT; (c) PyBOP, DIPEA, DMF, RT or i. EDC, NHS-OH, DMF, DIPEA ii. NEt_3, DMF; (d) $SO_3{\cdot}NMe_3$, DMF, 60 °C, 72 h.

in the same assay to be 39 nM, a slight increase from previously reported values and likely caused by assay variability between laboratories. It can therefore be concluded that the trimer glycolipids are slightly less effective inhibitors of HPSE than Tet-29 (11 nM). However, there is not a substantial decrease in anti-heparanase activity despite a dendrimer arm having been removed, as the compounds remain nanomolar inhibitors of HPSE. Trimer **6j**, with an IC_{50} for inhibition of HPSE of 87 nM, was selected for further study.

In vitro assay: tube formation

The ability of human umbilical vascular endothelial cells (HUVEC) to show capillary-like tube formation on basement membrane gel extract is considered a reliable replicator of angiogenesis *in vitro*.[31] Both HPSE and HS are implicated in promoting angiogenesis – HPSE modulates release and activity of HS–sequestered growth factors, adhesion receptors, and ECM components.[18b] The effect of HS mimetics on tube formation was measured to determine their anti-angiogenic potential (Figure 2, B&C). Both Tet-29 and trimer glycolipid **6j** showed anti-angiogenic activity, with both reducing tube formation at 3 and 10 μM. The mechanism of action appears to be somewhat different to that of the positive control, docetaxel, as cells treated with **6j** or Tet-29 exhibited increased clumping in a dose-dependent manner, suggesting that these compounds may interfere with cell migration and/or cell-matrix adhesion rather than inducing apoptosis.

Both compounds reducing tube formation indicates that anti-angiogenic activity could be a potentially important mechanism of their anticancer affect, although future *in vivo* studies are required to confirm this.

In vitro assay: MTT cell viability assay

The compounds selected for *in vivo* studies, **6j** and Tet-29, were first tested for potential cytotoxic effects against tumor cells using the MTT cell viability assay. Neither of the compounds displayed any cytotoxic effect *in vitro* at concentrations of up to 40 μM against MC38 murine colon adenocarcinoma cells or HT29 human colorectal adenocarcinoma cells (Suppl. Figure 1, Suppl. Info.).

Molecular modelling of a HS fragment and the Tet-29 capping group interactions with heparanase

In order to investigate mimetic-HPSE interactions we modelled both a HS fragment and Tet-29 capping group (Figure 3, A) to the active site of heparanase using extended sampling Induced Fit Docking[32] (IFD) and MM-GBSA (Molecular Mechanics Generalized Born Surface Area) scoring.[33] The best scored pose

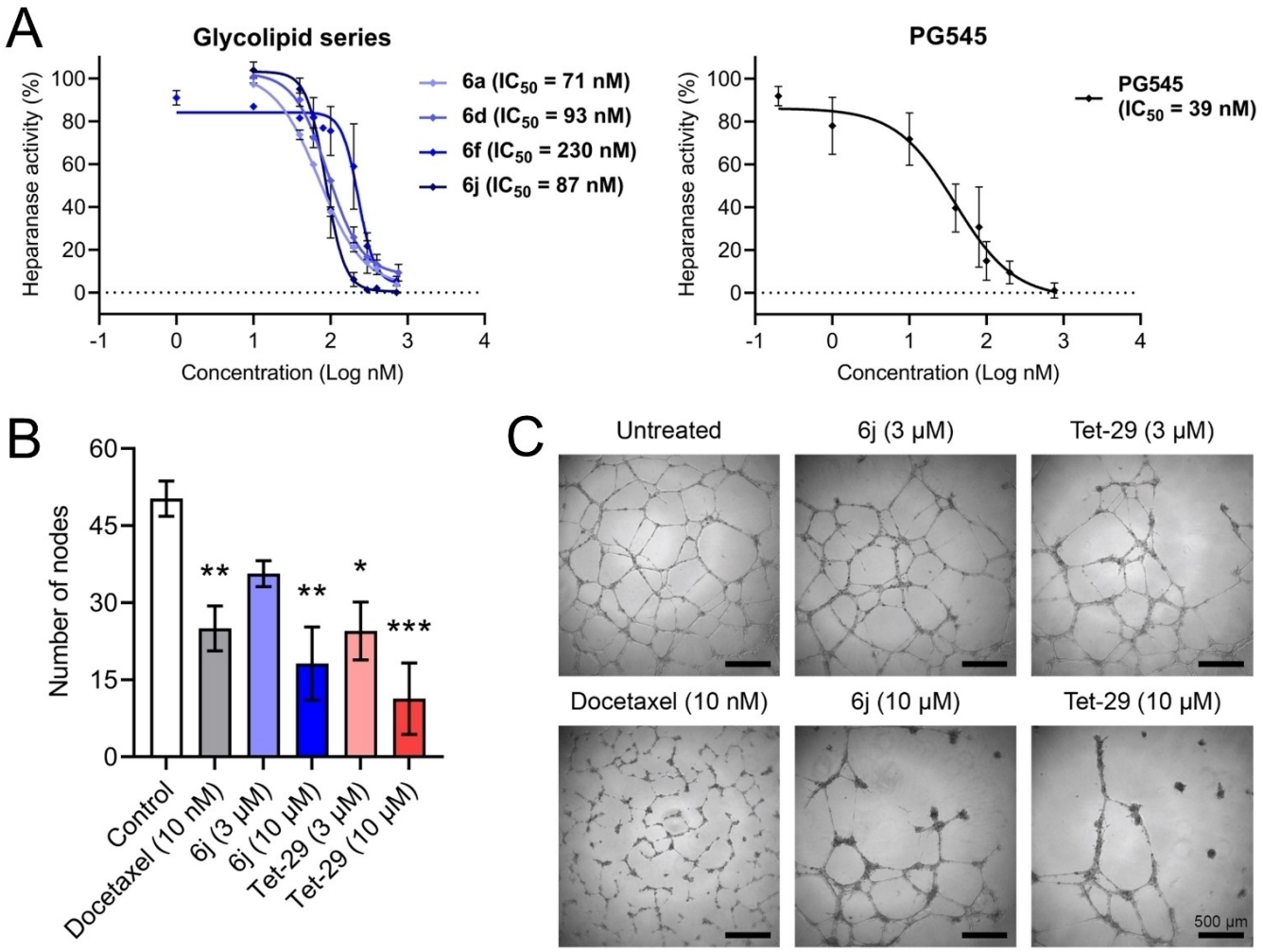


Figure 2. (A) HPSE inhibition by HS mimetics with IC_{50} values in the low nanomolar range. (B and C) Effect of the HS mimetics on ability of HUVEC to show capillary-like tube formation on basement membrane gel extract extract. Both Tet-29 and trimer glycolipid **6j** exhibited anti-angiogenic activity, with both reducing HUVEC tube formation at 3 and 10 μM. Statistical analysis was performed using one-way ANOVA with post-hoc Dunnett test to correct for multiple comparisons, comparing various treatments to the untreated control. Representative images of HUVEC tube formation are shown in C. All scale bars depict 500 μm. All data are representative of three independent experiments. Graphs represent the mean and standard error of the mean (SEM, error bars). * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

of the HS fragment showed salt bridge interactions with Lys159, Lys231, Arg272, Lys274, and Arg303, and hydrogen bonds with Asn64, Thr97, Lys159, Glu225, Lys231, Gln270, Arg272, Tyr298, Arg303, Glu343, and Tyr391 (Figure 3, B,C). The capping group of Tet-29 was predicted to bind to the same active site region as the HS fragment and interacts with a similar set of residues (Figure 3, C). A MM-GBSA binding energy of −92.9 kcal/mol was calculated for the HS fragment, whereas the dendron capping group was calculated with a more favourable MM-GBSA binding energy at −97.8 kcal/mol, consistent with the observed inhibitory potency of Tet-29. The modelling results suggest that despite the capping group containing fewer sugar residues than the HS fragment, these compounds likely bind to the same region as the native substrate. That they do so with slightly better affinity than the HS fragment indicates the compounds could potentially act as competitive inhibitors. Furthermore, these calculations will assist in future compound design and lay the foundations for further work in the complex field of carbohydrate structure-activity-relationships.

In vivo studies

In our previous work, Tet-29 was identified from a library of HS-glycomimetics based on its ability to potently inhibit HPSE *in vitro*, while an analogous tetramer compound was shown to reduce tumor growth and metastasis in a xenograft mouse model of human myeloma.[29] Based on its simplified structure, Tet-29 was selected for further assessment in the MC38 murine model of colon adenocarcinoma. Briefly, MC38 tumor cells were subcutaneously implanted into the right hind flank of syngeneic C57BL/6 mice, that were then treated with 600 μg Tet-29 daily or every two days for a total duration of 40 days (Figure 4, A). Tet-29 was administered by intraperitoneal (i.p.) injection, with mice receiving i.p. injections of Dulbecco's

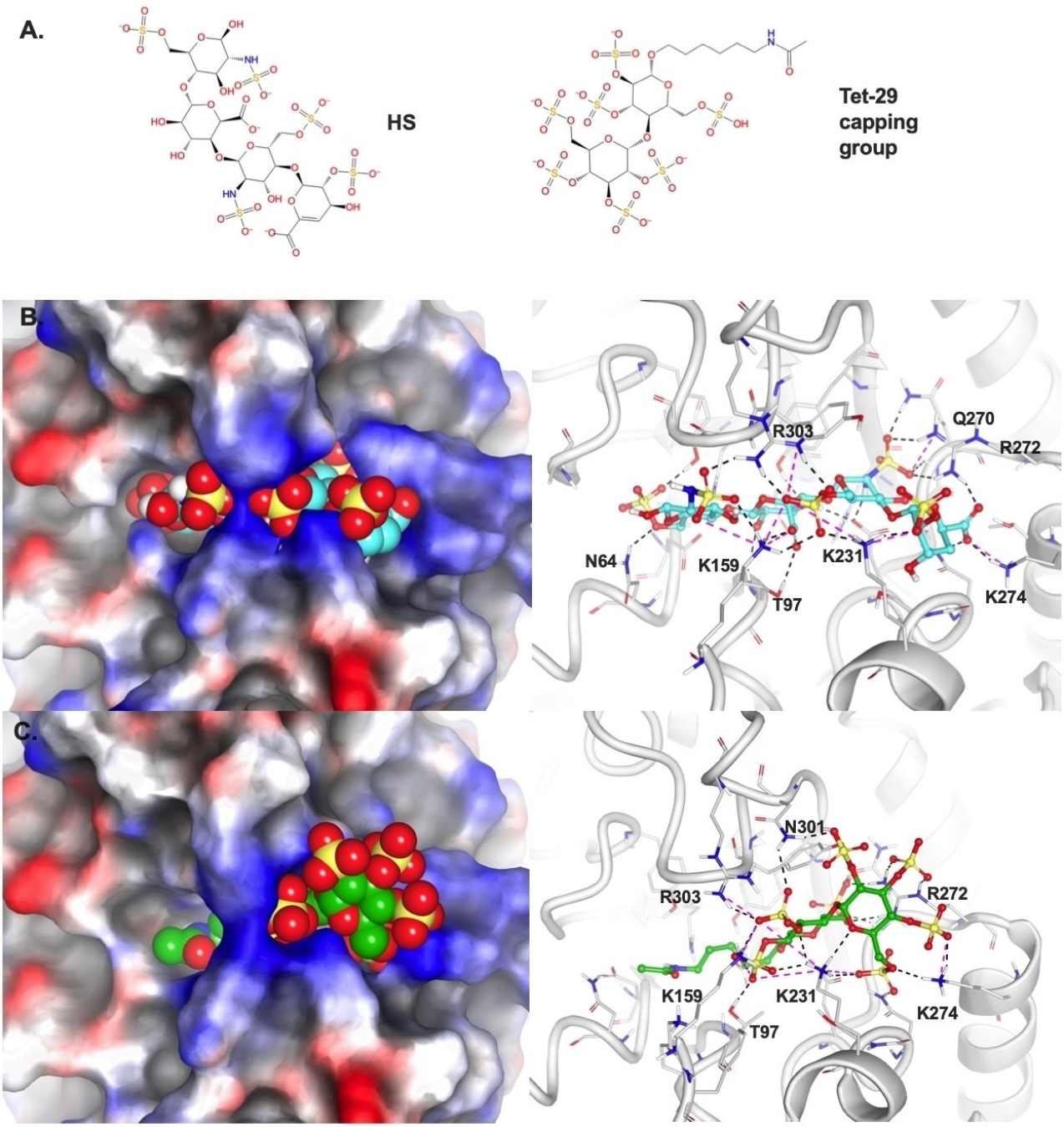


Figure 3. (A) Structures of a HS fragment and the Tet-29 capping group used in the modelling calculations. (B) Left: predicted binding conformation of the HS fragment (carbons are cyan) to the active site of heparanase (shown in electrostatic potential surface representation with blue-white-red color scale representing positive-neutral-negative electrostatic potentials respectively). Right: Salt bridge (magenta dashed lines) and hydrogen bonds (black dashed lines) established in the best scored binding pose of HS. (C) Left: predicted binding conformation of the Tet-29 capping group (carbons are green) to the active site of heparanase. Right: Salt bridge and hydrogen bonds established in the best scored binding pose of Tet-29 capping group.

Phosphate Buffered Saline (DPBS, vehicle) every second day as a negative control.

Strikingly, daily administration of Tet-29 greatly inhibited MC38 tumor growth and enhanced overall survival compared to sham-treated controls (Figure 4, B&C). Of note, only one mouse in the Tet-29 daily treatment group developed a large tumor ($>150\ mm^2$), with the remaining animals either not developing tumors at all or initially developing tumors that subsequently regressed. In contrast, Tet-29 had no influence on MC38 growth when administered every second day, with mice exhibiting poor overall survival and tumor growth which was comparable to sham-treated controls (Figure 4, B&C). Hence, Tet-29 only displayed therapeutic activity when administered daily, which may suggest a short half-life of this compound *in vivo*.

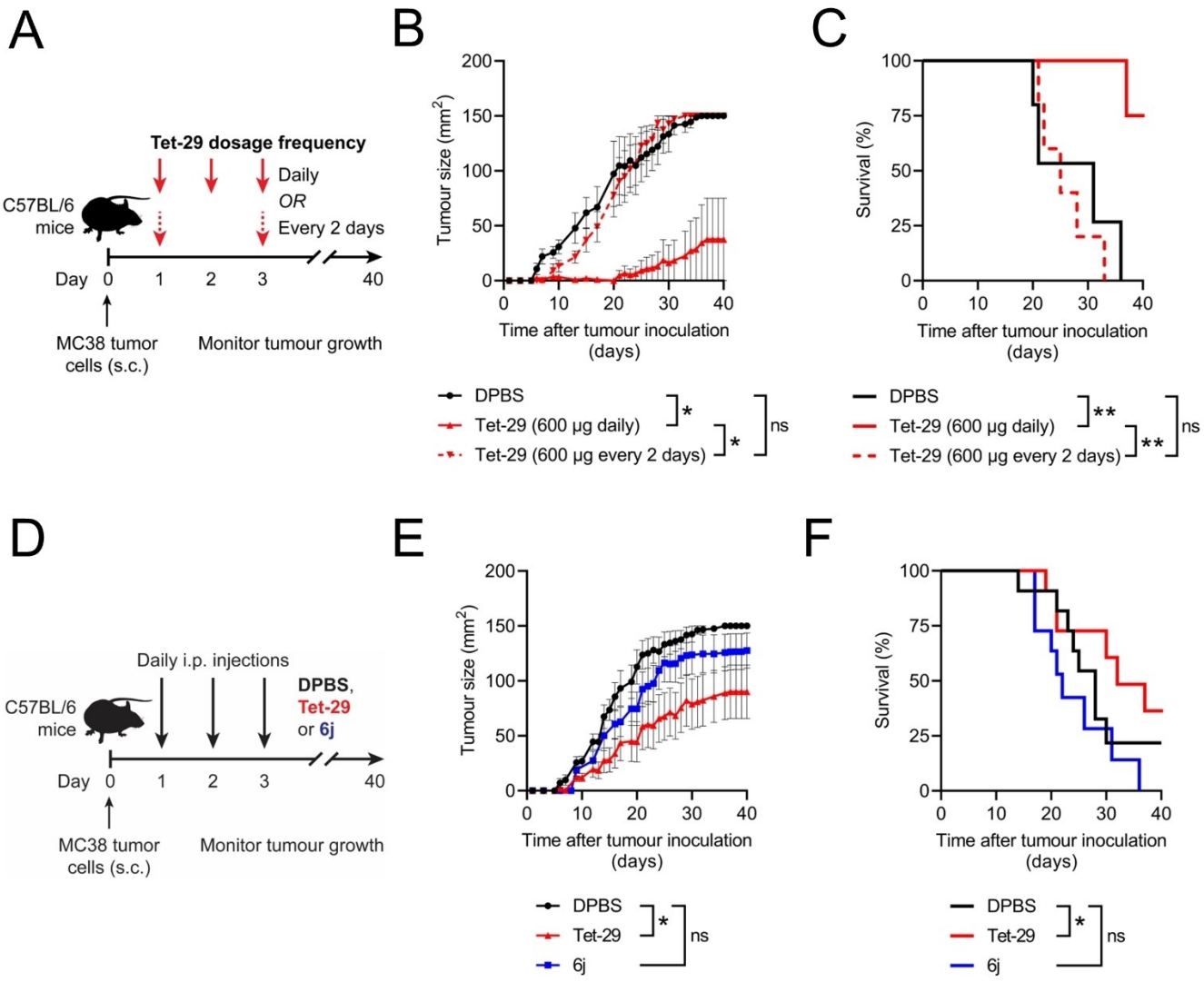


Figure 4. Therapeutic efficacy of novel HS mimetics in a murine model of colorectal cancer. (A) Schematic overview of tumor trial to assess Tet-29 dosage frequency. C57BL/6 mice (n = 4–5 mice per group) were inoculated with murine MC38 CRC cells (1×10^5 cells) by subcutaneous injection into the right hind flank (day 0). From day 1, mice were treated with 600 µg Tet-29, administered by intraperitoneal (i.p.) injection daily (solid red arrows) or every two days (dashed red arrows) for a total duration of 40 days. As a negative control, mice received sham treatment (i.p. injections of DPBS) every two days. (B) Tumor growth curves for each treatment group, depicting the mean (line) and standard error of the mean (SEM, error bars). (C) Overall survival of mice receiving Tet-29 daily or every two days. (D) Schematic overview of tumor trial administrations to compare the efficacy of novel HS mimetics. C57BL/6 mice (n = 8–10 mice per group) were inoculated with 1.5×10^5 MC38 murine tumor cells as described previously. From day 1, mice were treated daily with either Tet-29 or 6j (both 600 µg administered by i.p. injection) for a total duration of 40 days. As a negative control, mice received sham treatment (daily i.p. injections of DPBS). (E) Tumor growth curves for each treatment group, depicting the mean (line) and SEM (error bars). (F) Overall survival of mice receiving Tet-29 or 6j daily. Survival curves of treatment groups were compared to that of the sham treatment (DPBS) control (log-rank, Mantel-Cox test). Tumor growth curves were compared using one-way ANOVA with post-hoc Tukey test to correct for multiple comparisons. ns = not significant; * $p \leq 0.05$; ** $p \leq 0.01$.

Having established an *in vivo* model to examine the anti-tumor efficacy of HS mimetics, we moved towards a further simplification and optimisation with the development of the trimer glycolipid series to test whether any activity was retained in the trimer and whether bioavailability would be improved. A representative of this library (**6j**) was tested against Tet-29 in order to assess if the activity was comparable. This test was carried out in a colorectal cancer (CRC) mouse model in order to expand the library of cancers that the class of compounds show activity against: myeloma, C57MG murine breast cancer and in the present study MC-38 murine CRC. Following tumor inoculation, mice were treated with either Tet-29 or **6j** using the optimal dosage and frequency of administration established previously (600 µg daily via i.p. injection; Figure 4, D). Again, mice received daily i.p. injections of DPBS as negative controls, and tumor growth was monitored for a total duration of 40 days (Figure 4, D).

Consistent with our previous observations, daily administration of Tet-29 significantly delayed tumor growth and improved overall survival compared to sham-treated controls (Figure 4, E&F). By comparison, mice that received daily administration of **6j** showed a modest trend of delayed tumor growth; however, this was not statistically significant when compared to sham-treated controls (Figure 4, E). Similarly, there was no statistically

significant difference in overall survival between mice that received daily administration of **6j** and sham-treated controls (Figure 4, F). Hence, the tetramer provides superior anti-tumor efficacy *in vivo* to the trimer, possibly because of the reduced charge and multivalency of the trimer.

Conclusion

We have prepared a targeted library of novel single-entity trimer glycolipids presenting polyvalent displays of sulfated saccharides. Representatives of this library displayed low nanomolar heparanase inhibition despite a significant structural simplification and reduction in overall charge compared to the tetramer. The novel trimer glycolipid **6j** showed similar activity to Tet-29 in *in vitro* tube formation assays, with both reducing tube formation in the low micromolar range. Through docking and molecular modeling, we determined that the capping group of our glycomimetics is capable of binding to the same region of HPSE as a HS fragment. Finally, *in vivo* treatment of colorectal cancer in a mouse model showed that daily administration of trimer glycolipid **6j** produced a modest trend of delayed tumor growth that was not statistically significant when compared to sham-treated controls. This could be a result of the reduced valency of the trimer compared to Tet-29. Daily administration of Tet-29 significantly delayed tumor growth and improved overall survival indicating superior anti-tumor efficacy *in vivo*.

Acknowledgements

We gratefully acknowledge the financial support of the New Zealand Ministry of Business, Innovation and Employment (grant: RTVU1801), the New Zealand Breast Cancer Foundation (grant: R1703) and the Wellington Medical Research Foundation for help with the biological assays. We thank Dr. Andrew Lewis and Dr. Yinrong Lu for an exceptional NMR and mass spectroscopy service, and Ye Li for assistance with the synthesis. Furthermore, we would like to acknowledge Prof Vitto Ferro for generously supplying PG545 with which to perform in vitro experiments. Open access publishing facilitated by Victoria University of Wellington, as part of the Wiley - Victoria University of Wellington agreement via the Council of Australian University Librarians.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: Carbohydrates · Colorectal cancer · Glycolipids · Heparin · Heparan Sulfate · Molecular modelling

[1] D. Xu, J. D. Esko, *Annu. Rev. Biochem.* **2014**, *83*, 129–157.
[2] D. A. Simon Davis, C. R. Parish, *Front. Immunol.* **2013**, *4* (470), 1–7.
[3] S. Sarrazin, W. C. Lamanna, J. D. Esko, *Cold Spring Harbor Perspect. Biol.* **2011**, *3*, a004952.
[4] I. Vlodavsky, H. Q. Miao, B. Medalion, P. Danagher, D. Ron, *Cancer Metastasis Rev.* **1996**, *15*, 177–186.
[5] H. C. Christianson, M. Belting, *Matrix Biol.* **2014**, *35*, 51–55.
[6] a) J. R. Bishop, M. Schuksz, J. D. Esko, *Nature* **2007**, *446*, 1030–1037; b) J. Turnbull, A. Powell, S. Guimond, *Trends Cell Biol.* **2001**, *11*, 75–82.
[7] a) H. Lortat-Jacob, *Curr. Opin. Struct. Biol.* **2009**, *19*, 543–548; b) Z. Scholefield, E. A. Yates, G. Wayne, A. Amour, W. McDowell, J. E. Turnbull, *J. Cell Biol.* **2003**, *163*, 97–107.
[8] G. Torri, A. Naggi, *Int. J. Cardiol.* **2016**, *212*, S1-S4.
[9] U. Lindahl, J. P. Li, *J. Thromb. Haemostasis* **2020**, *18*, 2422–2424.
[10] a) S. Poon, X. Lu, R. A. A. Smith, P. Ho, K. Bhakoo, V. Nurcombe, S. M. Cool, *Angiogenesis* **2018**, *21*, 777–791; b) G. A. McCanney, M. A. McGrath, T. D. Otto, R. Burchmore, E. A. Yates, C. D. Bavington, H. J. Willison, J. E. Turnbull, S. C. Barnett, *Glia* **2019**, *67*, 668–687.
[11] X. M. van Wijk, T. H. van Kuppevelt, *Angiogenesis* **2014**, *17*, 443–462.
[12] V. Nurcombe, S. M. Cool, *Crit. Rev. Eukaryotic Gene Expression* **2007**, *17*, 159–171.
[13] J. M. Whitelock, R. V. Iozzo, *Chem. Rev.* **2005**, *105*, 2745–2764.
[14] R. Schwörer, O. V. Zubkova, J. E. Turnbull, P. C. Tyler, *Chem. Eur. J.* **2013**, *19*, 6817–6823.
[15] A. Ori, M. C. Wilkinson, D. G. Fernig, *J. Biol. Chem.* **2011**, *286*, 19892–19904.
[16] I. Vlodavsky, N. Ilan, R. D. Sanderson, *Adv. Exp. Med.* **2020**, *1221*, 3–59.
[17] I. Vlodavsky, P. Singh, I. Boyango, L. Gutter-Kapon, M. Elkin, R. D. Sanderson, N. Ilan, *Drug Resist. Updates* **2016**, *29*, 54–75.
[18] a) X. Hu, L. Zhang, J. Jin, W. Zhu, Y. Xu, Y. Wu, Y. Wang, H. Chen, K. A. Webster, H. Chen, H. Yu, J. Wang, *Stem Cells* **2015**, *33*, 1850–1862; b) S. Rivara, F. M. Milazzo, G. Giannini, *Future Med. Chem.* **2016**, *8*, 647–680.
[19] N. Ilan, M. Elkin, I. Vlodavsky, *Int. J. Biochem. Cell Biol.* **2006**, *38*, 2018–2039.
[20] K. M. Jayatilleke, M. D. Hulett, *J. Transl. Med.* **2020**, *18*, 453.
[21] N. Ilan, A. Shteingauz, I. Vlodavsky, *Autophagy* **2015**, *11*, 2387–2389.
[22] L. Gutter-Kapon, D. Alishekevitz, Y. Shaked, J.-P. Li, A. Aronheim, N. Ilan, I. Vlodavsky, *Proc. Nat. Acad. Sci.* **2016**, *113* (48), E7808-E7817.
[23] I. Vlodavsky, M. Gross-Cohen, M. Weissmann, N. Ilan, R. D. Sanderson, *Trends Biochem. Sci.* **2018**, *43*, 18–31.
[24] L. Wu, C. M. Viola, A. M. Brzozowski, G. J. Davies, *Nat. Struct. Mol. Biol.* **2015**, *22*, 1016–1022.
[25] a) D. Pala, L. Scalvini, G. M. Elisi, A. Lodola, M. Mor, G. Spadoni, F. F. Ferrara, E. Pavoni, G. Roscilli, F. M. Milazzo, G. Battistuzzi, S. Rivara, G. Giannini, *J. Enzyme Inhib. Med. Chem.* **2020**, *35*, 1685–1696; b) V. N. Madia, A. Messore, L. Pescatori, F. Saccoliti, V. Tudino, A. De Leo, M. Bortolami, L. Scipione, R. Costi, S. Rivara, L. Scalvini, M. Mor, F. F. Ferrara, E. Pavoni, G. Roscilli, G. Cassinelli, F. M. Milazzo, G. Battistuzzi, R. Di Santo, G. Giannini, *J. Med. Chem.* **2018**, *61*, 6918–6936.
[26] a) H. Zhou, S. Roy, E. Cochran, R. Zouaoui, C. L. Chu, J. Duffner, G. Zhao, S. Smith, Z. Galcheva-Gargova, J. Karlgren, N. Dussault, R. Y. Kwan, E. Moy, M. Barnes, A. Long, C. Honan, Y. W. Qi, Z. Shriver, T. Ganguly, B. Schultes, G. Venkataraman, T. K. Kishimoto, *PLoS One* **2011**, *6*, e21106; b) C. J. Liu, P. H. Lee, D. Y. Lin, C. C. Wu, L. B. Jeng, P. W. Lin, K. T. Mok, W. C. Lee, H. Z. Yeh, M. C. Ho, S. S. Yang, C. C. Lee, M. C. Yu, R. H. Hu, C. Y. Peng, K. L. Lai, S. S. Chang, P. J. Chen, *J. Hepatol.* **2009**, *50*, 958–968; c) J. P. Ritchie, V. C. Ramani, Y. Ren, A. Naggi, G. Torri, B. Casu, S. Penco, C. Pisano, P. Carminati, M. Tortoreto, F. Zunino, I. Vlodavsky, R. D. Sanderson, Y. Yang, *Clin. Cancer Res.* **2011**, *17*, 1382.
[27] V. Ferro, L. Liu, K. D. Johnstone, N. Wimmer, T. Karoli, P. Handley, J. Rowley, K. Dredge, C. P. Li, E. Hammond, K. Davis, L. Sarimaa, J. Harenberg, I. Bytheway, *J. Med. Chem.* **2012**, *55*, 3804–3813.
[28] P. C. Tyler, S. E. Guimond, J. E. Turnbull, O. V. Zubkova, *Angew. Chem. Int. Ed.* **2015**, *54*, 2718–2723; *Angew. Chem.* **2015**, *127*, 2756–2761.
[29] O. V. Zubkova, Y. A. Ahmed, S. E. Guimond, S.-L. Noble, J. H. Miller, R. A. Alfred Smith, V. Nurcombe, P. C. Tyler, M. Weissmann, I. Vlodavsky, J. E. Turnbull, *ACS Chem. Biol.* **2018**, *13*, 3236–3242.
[30] a) M. Chhabra, V. Ferro, *Molecules* **2018**, *23*; b) E. Hammond, C. P. Li, V. Ferro, *Anal. Biochem.* **2010**, *396*, 112–116.

[31] K. L. Au-DeCicco-Skinner, G. H. Au-Henry, C. Au-Cataisson, T. Au-Tabib, J. C. Au-Gwilliam, N. J. Au-Watson, E. M. Au-Bullwinkle, L. Au-Falkenburg, R. C. Au-O'Neill, A. Au-Morin, J. S. Au-Wiest, *JoVE* **2014**, *91*, e51312, 1–8.
[32] a) R. Farid, T. Day, R. A. Friesner, R. A. Pearlstein, *Bioorg. Med. Chem.* **2006**, *14*, 3160–3173; b) W. Sherman, T. Day, M. P. Jacobson, R. A. Friesner, R. Farid, *J. Med. Chem.* **2006**, *49*, 534–553; c) W. Sherman, H. S. Beard, R. Farid, *Chem. Biol. Drug Des.* **2006**, *67*, 83–84; d) S. Schrödinger Release 2018–4: Induced Fit Docking protocol; Glide, LLC, New York, NY, 2018; Prime, Schrödinger, LLC, New York, NY, 2018.
[33] a) M. P. Jacobson, D. L. Pincus, C. S. Rapp, T. J. Day, B. Honig, D. E. Shaw, R. A. Friesner, *Proteins* **2004**, *55*, 351–367; b) M. P. Jacobson, R. A. Friesner, Z. Xiang, B. Honig, *J. Mol. Biol.* **2002**, *320*, 597–608.

Manuscript received: March 3, 2022
Revised manuscript received: April 11, 2022
Accepted manuscript online: April 15, 2022
Version of record online: April 29, 2022