Available online at www.sciencedirect.com
ScienceDirect
Carbohydrate Polymers 67 (2007) 260–264
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

Short communication

Inhibitory effects of glycopolymers having globotriose and/or lactose on cytotoxicity of Shiga toxin 1

Atsushi Miyagawa, Maria Carmelita Z. Kasuya, Kenichi Hatanaka *

Institute of Industrial Science, The University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153-8505, Japan

Received 16 March 2006; received in revised form 19 May 2006; accepted 26 May 2006
Available online 18 July 2006

Abstract

Glycopolymers having globotriose and/or lactose were synthesized and evaluated for the inhibitory effects on cytotoxicity of Shiga toxins (Stxs; Stx1 and Stx2). Results showed that glycopolymers having globotriose units exhibited inhibitory effect on cytotoxicity of Stx1 while glycopolymers having small amount of acrylamide exhibited inhibitory effect on cytotoxicity of Stx2. Surprisingly, the glycopolymer having lactose showed inhibitory effect on cytotoxicity of Stx1. The binding of the glycopolymers to Stx1 was also investigated by using BIAcore system. The results showed that the glycopolymer having lactose, which is not known as ligand of Stxs, binds to Stx1. Therefore, Stx1 also recognized the internal residues (Galβ(1–4)Glcβ) of Gb3 (Galα(1–4)Galβ(1–4)Glcβ-ceramide) by composing a cluster. This result suggests that the cluster of the carbohydrates, which have very weak binding affinity for the receptor, can interact with the receptor.

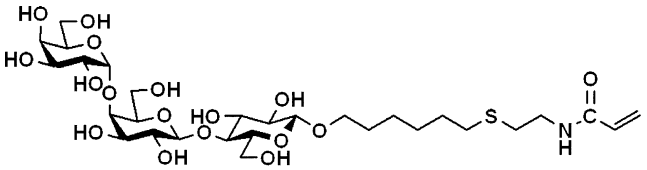
Keywords: Glycopolymer; Lactose; Shiga toxin; Globotriose; Carbohydrate recognition

Shiga toxins (Stxs; Stx1 and Stx2) produced by pathogenic *Escherichia coli* (STEC) cause diarrhea, hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), and neurological damage in humans. The Stxs are categorized as a family of AB5 subunit toxins. The enzymatic A subunit (32 kDa), which has RNA *N*-glycosidase activity, is non-covalently associated with the pentamer of receptor-binding B subunits (7.5 kDa) (Gariépy, 2001; Nishikawa et al., 2002). The carbohydrate receptor for Stxs had been studied by many researchers. Binding activities and inhibitory activities for Stxs of the receptors were provided with great details. *N*-Acetyl-D-glucosamine oligomers and oligosaccharides, which have Galα(1–4)Gal structure at the reducing end, potently inhibited cytotoxicity of Stx1. The highest active receptor of Stx1 is globotriaosyl ceramide [Gb3, Galα(1–4)Galβ(1–4)Glcβ-ceramide] (Boyd & Lingwood, 1989; Keusch & Jacewicz, 1977; Lindberg et al., 1978; Lingwood et al., 1978). During carbohydrate recognition, it is important for carbohydrates to cluster on the cell surface. The cluster of carbohydrates usually has a high binding constant for the carbohydrate-recognizing protein because of cluster effect. Hence, many kinds of glycopolymers, which can work as carbohydrate clusters, were synthesized and investigated for development of inhibitors and neutralizing agents. Glycopolymers having globotriose or galabiose, which contains the important Galα(1–4)Gal structure, were also reported as neutralizing agents for Stxs. Glycopolymer having globotriose showed stronger binding activity and neutralizing activity than glycopolymer having galabiose (Armstrong, Fodor, & Vanmaele, 1991). However, it was reported that glycopolymer having lactose, which has Galβ(1–4)Glc structure at non-reducing end of Gb3, did not affect the cytotoxicity of Stxs (Dohi et al., 1999; Watanabe et al., 2004). We describe here the inhibitory effect of a glycopolymer having lactose on cytotoxicity of Stx1. Concurrently, glycopolymers having Gb3 or cellobiose were evaluated for their inhibitory activity for Stx1. Furthermore, by using BIAcore system, bindings of Stx1 for glycopolymers were observed.

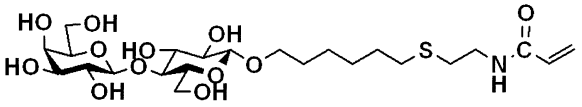
The glycopolymers were synthesized and their polymer compositions and molecular weights were analyzed as

* Corresponding author. Tel.: +81 3 5452 6355; fax: +81 3 5452 6356.
E-mail address: hatanaka@iis.u-tokyo.ac.jp (K. Hatanaka).

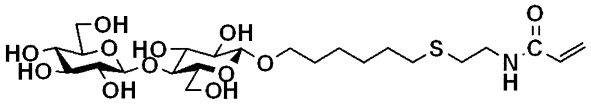
0144-8617/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2006.05.020



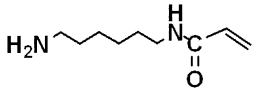
Gb3 monomer (1)



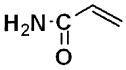
Lac monomer (2)



Cel monomer (3)



Amine monomer (4)



Acrylamide (5)
(AA)

Fig. 1. Monomer structures.

shown in Table 1. Then, these polymers were immobilized on cellulose materials (Miyagawa, Kasuya, Matsuoka, & Hatanaka, 2003). While, in this paper, these polymers were used in a solution. These polymers were evaluated according to the inhibitory effects on cytotoxicity of Stxs. The results are shown in Fig. 2. Polymer (Gb3:AA = 1:0) was highly active for both Stx1 and Stx2, while polymers (Gb3:AA = 1:5) and (Gb3:AA = 1:9.5) showed lower inhibitory effects for Stx1 than that of polymer (Gb3:AA = 1:0). Surprisingly, polymer (Lac:AA = 1:0) had the same activity as polymers (Gb3:AA = 1:5) and (Gb3:AA = 1:9.5). It was known that lactose is not the ligand of Stxs though lactose has Galβ(1–4)Glc structure at the non-reducing end of Gb3. On the other hand, the polymer (Cel:AA = 1:0) having cellobiose, which is not the ligand of Stxs, showed no activity in the same evaluation. The evaluation result of the polymer (Cel:AA = 1:0) indicated that the polymer (Lac:AA = 1:0) has potentiality as the ligand of Stx1.

Hence, these polymers were investigated for the binding affinity of Stx1 by using BIAcore system. The glycopolymers were immobilized on the sensor chip and evaluated for binding with Stx1. The results are shown in Fig. 3. Stx1 strongly bound to polymers (Gb3:AA = 1:0) and (Gb3:AA = 1:5) and slightly bound to (Lac:AA = 1:0). These results do not suggest that the polymer (Lac:AA = 1:0) inhibited the cytotoxicity of Stx1.

Consequently, different kinds of polymers were synthesized and evaluated for the inhibitory effect on cytotoxicity of Stxs. The compositions of the polymers are shown in Table 2 and the evaluations of the inhibition of Stxs by the polymers are shown in Fig. 4. Gb3 series of polymers were improved by changing the ratio of acrylamide and glycosyl monomer. As shown in Figs. 2 and 4, polymers (Gb3:AA = 1:1.6) and (Gb3:AA = 1:3) showed stronger inhibition than those of (Gb3:AA = 1:5) and (Gb3:AA = 1:9.5). Glycopolymers, which were copolymerized with acrylamide, had low solubility. Therefore, Gb3 monomer was copolymerized with lactose monomer and evaluated to improve solubility. Polymers (Gb3:Lac = 1:3.9) and (Gb3:Lac = 1:9.6) showed a little stronger inhibition than those of glycopolymer copolymerized with acrylamide. By copolymerizing with lactose monomer, the glycopolymers became to have high solubility and Gb3 in the glycopolymers was exposed to Stx1, like being bound better. The polymer (Lac:AA = 1:0) showed inhibitory effect on Stx1, while polymers (Lac:AA = 1:5.3) and (Lac:AA = 1:8.4), which are copolymers with acrylamide, had no activity. From this result, it was found that only homopolymer (Lac:AA = 1:0), had inhibitory effect on cytotoxicity of Stx1. Stx1 would simultaneously recognize several lactose units

Table 1
Results of copolymerization

Polymer	Monomer ratio							
	Glycosyl monomer							
	Gb3 monomer (**1**)	Lac monomer (**2**)	Cellobiose monomer (**3**)	Amine monomer (**4**)	Acryl-amide (**5**)	Total yield (%)	Polymer composition[a]	Mw[b] (kDa)
(Gb3:AA = 1:0)	1.0	—	—	0.05	—	89.0	1 : 0.13	248
(Gb3:AA = 1:5)	1.0	—	—	0.05	4.0	84.9	1 : 0.03 : 5.0	644
(Gb3:AA = 1:9.5)	1.0	—	—	0.05	8.0	99.0	1: 0.05: 9.5	749
(Lac:AA = 1:0)	—	1.0	—	0.05	—	93.1	1 : 0.08	302
(Cel:AA = 1:0)	—	—	1.0	0.05	—	82.8	1 : 0.06	400

[a] The polymer composition was determined from the integration value of ^{1}H NMR.
[b] Mws were estimated by the SEC method with the TOSOH TSKgel G-Oligo-PW column, TSKgel G2500PWXL column, TSKgel G3000PWXL column and TSKgel G4000PWXL column [pullulans (5.8, 12.2, 23.7, 48.0, 100, 186, and 380 kDa, Shodex Standard P-82) were used as standards].

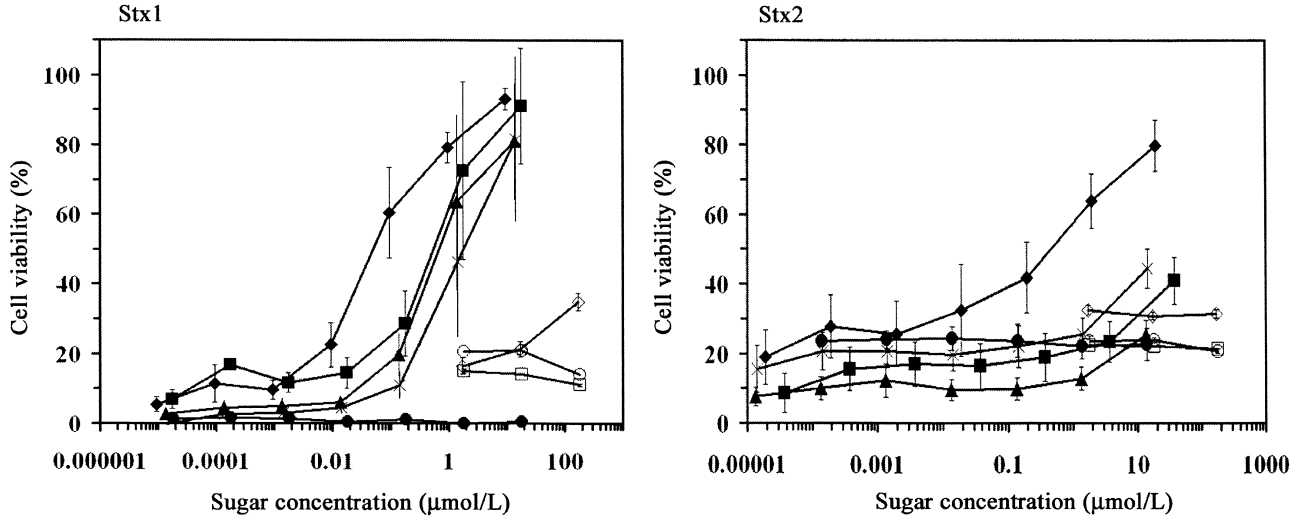


Fig. 2. Inhibitory effect of glycopolymers on biological activities of Shiga toxins in Vero cells. Data are presented as the percentage of cell viability in the absence of Stxs (means ± SE; $n = 3$ or 6). Filled diamonds, (Gb3:AA = 1:0); filled rectangles, (Gb3:AA = 1:5); filled triangles, (Gb3:AA = 1:9.5); filled circles, (Cel:AA = 1:0); asterisks, (Lac:AA = 1:0); open diamonds, hexenyl Gb3; open rectangles, Lac monomer; open circles, Cel monomer.

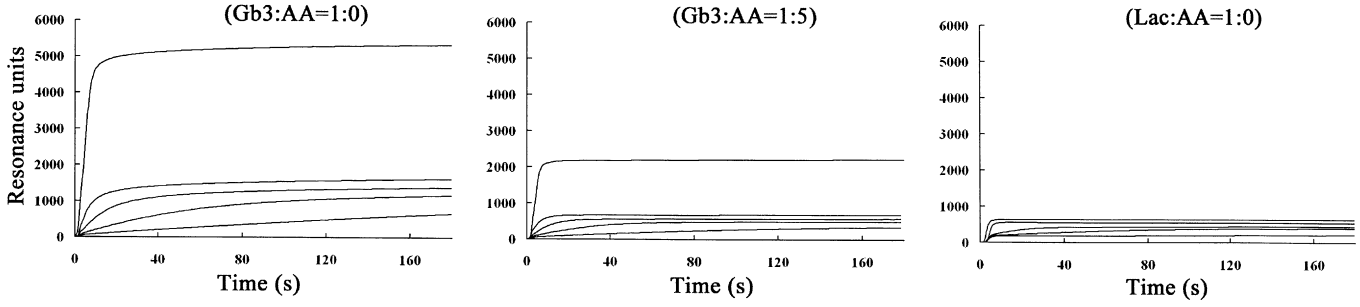


Fig. 3. Kinetic analysis of Stx1 binding to immobilized glycopolymers using BIAcore systems. Glycopolymers were immobilized on sensor chip CM5, and 100, 30, 10, 3, and 1 mg/ml of Stx1 were injected over each of the immobilized glycopolymers at a flow rate of 20 μl/min.

and afford affinity for lactose. Therefore, the carbohydrate-binding sites of Stx1 recognized also the internal residues (Galβ(1–4)Glcβ) of Gb3 (Galα(1–4)Galβ(1–4)Glcβ-ceramide) by composing the cluster.

Consequently, it was important for recognition of Stx1 that carbohydrates of ligand assemble at very high density. This result suggests that the cluster of the carbohydrates, which have just very weak binding affinity for receptor, might be an inhibitory agent. The inhibitory effects of glycopolymers can be used for the detection of weak and specific "carbohydrate–protein interaction".

Appendix A. Experimental

A.1. Materials

Unless otherwise stated, all commercially available solvents and reagents were used without further purification.

Table 2
Results of copolymerization

Polymer	Monomer ratio						
	Glycosyl monomer						
	Gb3 monomer (**1**)	Lac monomer (**2**)	Amine monomer (**4**)	Acryl-amide (**5**)	Total yield (%)	Polymer composition[a]	Mw[b] (kDa)
(Gb3:AA = 1 : 1.6)	1.0	—	0.05	1.0	77.3	1 : 0.04 : 1.6	131
(Gb3:AA = 1 : 3)	1.0	—	0.05	2.0	79.9	1 : 0.04 : 3.0	135
(Gb3:Lac = 1 : 3.9)	1.0	4.0	0.1	—	98.0	1: 3.9 : 0.05	194
(Gb3:Lac = 1 : 9.6)	1.0	8.0	0.1	—	98.5	1 : 9.6 : 0.06	271
(Lac:AA = 1 : 5.3)		1.0		2.0	47.4	1 : 5.3	90
(Lac:AA = 1 : 8.4)	—	1.0	0.1	8.0	50.2	1 : 8.4 : 0.07	400

[a] The polymer composition was determined from the integration value of ^{1}H NMR.

[b] Mws were estimated by the SEC method with the TOSOH TSKgel G-Oligo-PW column, TSKgel G2500PWXL column, TSKgel G3000PWXL column and TSKgel G4000PWXL column [pullulans (5.8, 12.2, 23.7, 48.0, 100, 186, and 380 kDa, Shodex Standard P-82) were used as standards].

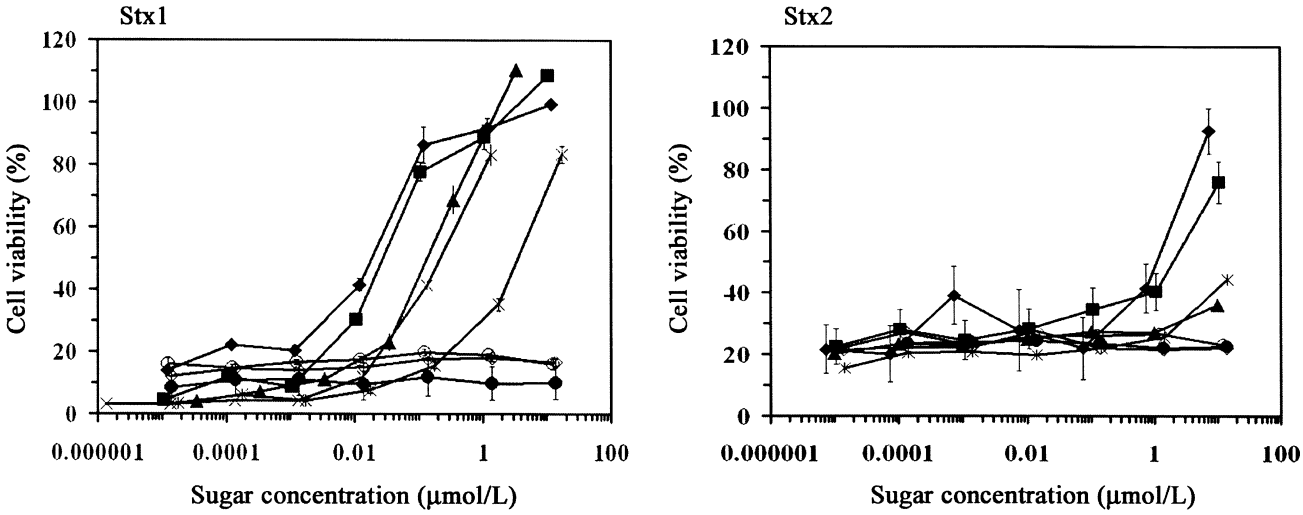


Fig. 4. Inhibitory effect of glycopolymers on biological activities of Shiga toxins in Vero cells. Data are presented as the percentage of cell viability in the absence of Stxs (means ± SE; $n = 3$ or 6). Filled diamonds, (Gb3:AA = 1:1.6); filled rectangles, (Gb3:AA = 1:3); filled triangles, (Gb3:Lac = 1:3.9); crosses, (Gb3:Lac = 1:9.6); asterisks, (Lac:AA = 1:0); open circles, (Lac:AA = 1:5.3); open diamonds, (Gb3:AA = 1:8.4); filled circles, (Cel:AA = 1:0).

Acrylamide was recrystallized from benzene. Stx1 and Stx2 were prepared according to methods described elsewhere (Noda, Yutsudo, Nakabayashi, Hirayama, & Takeda, 1987).

A.2. General methods

^{1}H NMR spectra were recorded at 400 or 600 MHz using a Jeol JNM-AL400 or Jeol ECP-600 spectrometer in chloroform-*d* or deuterium oxide. ^{13}C NMR spectra were recorded at 100.6 or 150.9 MHz with the same instruments. Assignments in the NMR spectra were made by first-order analysis of spectra, and supported by correlation spectroscopy and heteronuclear chemical shift correlation. The average molecular weights of the polymers were estimated by size exclusion chromatography (SEC) using a TOSOH TSKgel G-Oligo-PW column, TSKgel G2500PWXL column, TSKgel G3000PWXL column, and TSKgel G4000PWXL column with pullulans (5.8, 12.2, 23.7, 48.0, 100, 186, and 380 kDa, Shodex Standard P-82) as standards. Reactions were monitored by thin-layer chromatography (TLC) on a precoated plate of silica gel 60 F254 (layer thickness, 0.25 mm; E. Merk, Darmstadt, Germany). For detection of intermediates, TLC sheets were dipped in a solution of 85:10:5 (v/v/v) methanol-*p*-anisaldehyde-concentrated sulfuric acid and heated for a few minutes (for carbohydrates).

A.3. Polymerization

A solution of the glycosyl monomer (globotriose, lactose, or cellobiose), *N*-(6-aminohexyl)acrylamide, and acrylamide (Fig. 1) in deionized water was degassed using a diaphragm pump, and TEMED (0.1 or 0.2 equiv for glycosyl monomer) and APS (0.04 or 0.08 equiv for glycosyl monomer) were added to the solution. The reaction mixture was continuously stirred overnight at room temperature. The resulting product was purified by reprecipitation with a mixed solution of methanol and ethanol, and lyophilized to give a water-soluble copolymer as a powder. The monomer ratios are shown in Table 1.

A.4. Cells

Vero cells were maintained in DMEM supplemented with 10% fetal calf serum. Cells were seeded in 96-well plastic microplate for cytotoxicity assays.

A.5. Inhibition assay on cytotoxicity

Subconfluent Vero cells in a 96-well plate were treated with Stx1 or Stx2 (10 pg/ml) in the presence or absence of the desired amount of glycoconjugate polymer for 72 h. Relative cell number was determined using a WST-8 Cell Counting Kit (Wako Pure Industries).

A.6. Kinetic analysis of binding of Stxs to immobilized glycoconjugate polymers

The attachment of Stx1 on the glycoconjugate polymers were quantified by using a BIAcore system instrument (Pharmacia Biosensor, Uppsala, Sweden). The glycoconjugate polymer (10 μg/ml) was injected into the system to become immobilized on CM5 sensor chip. Various concentrations of Stx1 were injected (time 0) over the immobilized glycoconjugate polymer at a flow rate of 20 μl/min for at least 5 min to reach a plateau at 25 °C.

References

Armstrong, G. D., Fodor, E., & Vanmaele, R. (1991). Investigation of Shiga-like toxin binding to chemically synthesized oligosaccharide sequences. *Journal of Infectious Diseases, 164*, 1160–1167.

Boyd, B., & Lingwood, C. (1989). Verotoxin receptor glycolipid in human renal tissue. *Nephron, 51*, 207–210.

Dohi, H., Nishida, Y., Mizuno, M., Shinkai, M., Kobayashi, T., Takeda, T., et al. (1999). Synthesis of an artificial glycoconjugate polymer carrying

P^k-antigenic trisaccharide and its potent neutralization activity against Shiga-like toxin. *Bioorganic and Medical Chemistry, 7*, 2053–2062.

Gariépy, J. (2001). The use of Shiga-like toxin 1 in cancer therapy. *Critical Reviews in Oncology/Hematology, 39*, 99–106.

Keusch, G. T., & Jacewicz, M. (1977). Pathogenesis of *Shigella* diarrhea VII. Evidence for a cell membrane toxin receptor involving β1 → 4-linked *N*-acetyl-D-glucosamine oligomers. *The Journal of Experimental Medicine, 146*, 535–544.

Lindberg, A. A., Brown, J. E., Strömberg, N., Westling-Ryd, M., Schultz, J. E., & Karlsson, K.-A. (1978). Identification of the carbohydrate receptor for Shiga toxin produced by *Shigella dysenteriae* type 1. *The Journal of Biological Chemistry, 262*, 1779–1785.

Lingwood, C. A., Law, H., Richardson, S., Petric, M., Brunton, J. L., Grandis, S. D., et al. (1978). Glycolipid binding of purified and recombinant *Escherichia coli* produced Verotoxin *in vitro*. *The Journal of Biological Chemistry, 262*, 8834–8839.

Miyagawa, A., Kasuya, M.C., Matsuoka, K., & Hatanaka, K. (2003). Development of dialyzer as Verotoxin eliminator using glycoconjugate polymer. 12th European Carbohydrate Symposium (PD004).

Nishikawa, K., Matsuoka, K., Kita, E., Okabe, N., Mizuguchi, M., Hino, K., et al. (2002). A therapeutic agent with oriented carbohydrates for treatment of infections by Shiga toxin-producing *Escherichia coli* O157:H7. *Proceedings of the National Academy of Sciences of the United States of America, 88*, 7669–7674.

Noda, M., Yutsudo, T., Nakabayashi, N., Hirayama, T., & Takeda, Y. (1987). Purification and some properties of Shiga-like toxin from *Escherichia coli* O157: H7 that is immunologically identical to Shiga toxin. *Microbial Pathogenesis, 2*, 339–349.

Watanabe, M., Matsuoka, K., Kita, E., Igai, K., Higashi, N., Miyagawa, A., et al. (2004). Oral therapeutic agents with highly clustered globotriose for treatment of Shiga toxigenic *Escherichia coli* infections. *Journal of Infectious Diseases, 189*, 360–368.