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# Sulfated polymannuroguluronate, a novel anti-acquired immune deficiency syndrome (AIDS) drug candidate, targeting CD4 in lymphocytes

Benchun Miao[a], Meiyu Geng[a,1], Jing Li[a], Fuchuan Li[a], Haixia Chen[a], Huashi Guan[a], Jian Ding[b,*]

[a]*Department of Pharmacology, Marine Drug and Food Institute, Ocean University of China, Qingdao 266003, PR China*
[b]*Division of Antitumor Pharmacology, State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 201203, PR China*

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**Abstract**

Sulfated polymannuroguluronate (SPMG), a marine sulfated polysaccharide, has entered the Phase II clinical trial in China as the first anti-acquired immune deficiency syndrome (AIDS) drug candidate obtained from marine organisms. To determine the binding site(s) (receptors) of SPMG in lymphocytes mediating its anti-AIDS activities, fluorescein-5-isothiocyanate (FITC)-labeled SPMG was used to investigate SPMG binding to lymphocytes. Flow cytometry (FCM) and fluorescence microscopy analysis showed that the SPMG binds to lymphocytes in a rapid, specific, reversible, and saturable fashion. Several SPMG binding proteins were purified by affinity chromatography from lymphocyte membrane preparations. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting analysis revealed that a 55 kDa lymphocyte membrane protein is CD4. To characterize the SPMG and CD4 interaction, inhibition assay and surface plasmon resonance (SPR) assay were carried out. SPMG bound to CD4 in a multivalent fashion with specificity. The binding of SPMG to human lymphocyte CD4 was competitively inhibited by human soluble CD4 (hsCD4). Likewise, the binding between hsCD4 and immobilized SPMG was blocked by excess free SPMG. These results indicate that CD4 is one of the specific SPMG binding sites (receptors) in lymphocytes. The interaction between SPMG and CD4 may provide a mechanistic explanation of the immunopotentiating and anti-AIDS activities of SPMG in human immunodeficiency virus (HIV) infected individuals.


*Keywords:* SPMG; Lymphocytes; Binding sites (receptors); CD4; FCM; SPR

## 1. Introduction

There is a growing body of in vivo and in vitro evidence indicating that polysaccharides exert immunomodulating effects by stimulating both cellular and humoral immunoresponse [1–3]. The immunologic action of polysaccharides may begin with binding to effector cells such as lymphocytes, macrophages, natural killer (NK) cells and so on, which is followed by a series of signal transduction processes [4,5]. SPMG is a new form of sulfated polysaccharide extracted from *brown alga*. It is characterized by rich amount of 1,4-linked β-D-mannuronate with 1.02 sulfate and 1.0 carboxyl groups averaging each sugar residue (Fig. 1). SPMG has an average molecular weight at 8.0 kDa. SPMG has entered the Phase II clinical trial in China. It is therefore the first marine sulfated polysaccharide with the potential of becoming an anti-AIDS drug. Previous in vitro and in vivo studies from our group have demonstrated the SPMG inhibits HIV replication and the possible underlying cellular mechanisms may be due to the

*Abbreviations:* SPMG, sulfated polymannuroguluronate; AIDS, acquired immune deficiency syndrome; HIV, human immunodeficiency virus; FITC, fluorescein-5-isothiocyanate; BSA, bovine serum albumin; PMSF, phenyl methylsulphonyl fluoride; hsCD4, human soluble CD4; mAb, monoclonal antibody; FCM, flow cytometry; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SPR, surface plasmon resonance; HBS, HEPES-buffered saline

* Corresponding author. Tel.: +86 21 50806079; fax: +86 21 50806722.
*E-mail addresses:* gengmy@mail.ouc.edu.cn, suozhang@mail.shcnc.ac.cn (J. Ding).
[1] Co-corresponding author. Tel.: +86 532 2032066; fax: +86 532 2033054.

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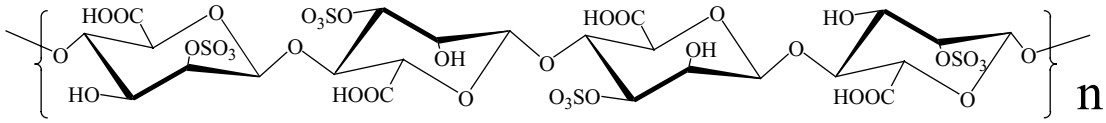


Fig. 1. The general structure of SPMG.

effect of SPMG in inhibiting the process of HIV entry [6,7]. The anti-HIV activity of SPMG may also be attributed to its immunopotentiating effects [8]. Numerous polysaccharides have been reported to exert immunomodulating activities by directly interacting with various receptors on the immunocyte surface [9,10]. Therefore, it is possible that there are specific SPMG binding sites (receptors) in immune cells. The goals of the present study are to investigate the binding of SPMG in lymphocytes and to identify and characterize the specific binding partner(s) or receptor(s).

## 2. Materials and methods

### 2.1. Drugs and reagents

SPMG has an average molecular weight at 8.0 kDa, SPMG-Sepharose and SPMG-FITC were provided by Marine Drug and Food Institute, Ocean University of China. FACS$^{TM}$ lysing solution, BSA, PMSF, Sulfo-NHS-biotin and streptavidin were purchased from Sigma. RPMI-1640 medium was provided by Gibco and hsCD4 was obtained from R&D Systems. Mouse anti-rat CD4 mAb (IgG) was purchased from Serotec. Horse anti-mouse IgG conjugated to alkaline phosphatase was from Vector Laboratories, and carboxymethylated dextran sensor chip (CM5) from Biacore.

### 2.2. Cell culture and preparation

Rat thymus and spleen lymphocytes were obtained from male Wistar rats (180–200 g) following published methods, and human lymphocytes were prepared by treating human peripheral blood provided by healthy volunteer using FACS$^{TM}$ lysing solution in dark for 5 min to lyse erythrocytes without activating the lymphocytes [11,12]. All cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) at 37 °C in humidified 5% $CO_2$ incubator.

### 2.3. FCM analysis

Rat thymus and spleen lymphocytes and human peripheral blood lymphocytes were seeded at a concentration of $5 \times 10^5$ cells per flask. FITC-labeled SPMG was added at a final concentration of 100 mg/L. After incubation for 2 h, the cells were harvested, washed with PBS for three times, and gated and analyzed by FCM (BD), with a 488 nm laser excitation and a 530 nm emission filter. An aliquot of each sample was spotted on a slide, analyzed and photographed under a fluorescence microscope (Olympus). To determine the binding kinetics, time- and dose-dependence, and temperature effect, cells were incubated with or without SPMG-FITC at concentrations ranging from 20 to 100 mg/L with incubation time varying from 0.5 to 2 h, and at 4, 25, or 37 °C. The reversibility and specificity of the binding were investigated as follows: after incubation of lymphocytes with SPMG-FITC for 1 h, unlabeled SPMG was added and incubated for another 1 h. The binding was examined by FCM. To determine the SPMG and CD4 binding, human lymphocytes ($5 \times 10^5$ per flask) were incubated with 1 mg/L SPMG-FITC together with or without hsCD4 at final concentrations of 25 and 50 mg/L for 2 h. The cells were collected, washed for three times with PBS, and $CD4^+$ T lymphocytes were gated and analyzed by FCM.

### 2.4. Purification of lymphocyte membrane proteins binding to SPMG

Lymphocyte membrane fraction was isolated from rat spleen and thymus tissues. The entire purification procedure was performed at 4 °C. The tissues were separated, minced, grinded, and filtrated. Lymphocytes were harvested in PBS and counted. Cells were centrifuged at $500 \times g$ for 5 min in a refrigerated centrifuge. Next, they were suspended in lysis buffer [10 mM Tris–HCl (pH 7.4), 1% Triton X-100 (v/v), 150 mM NaCl, 1 mM EDTA, 1 mM PMSF and 75 units/mL of aprotinin] and left for 20 min at 4 °C. The cells were dounce-homogenized with 10 strokes, and the lysate was centrifuged for 5 min at $1300 \times g$ to remove the nuclei and large cellular debris. The supernatant was collected and subjected to sucrose density gradient centrifugation at $7000 \times g$ for 90 min. The membrane fraction located at 37–41% (w/v) sucrose was harvested and washed for three times with 20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM PMSF by centrifugation at $100,000 \times g$ for 10 min [13–15].

To solublize the isolated membrane proteins, the membrane fractions were resuspended in solubilization buffer [20 mM Tris–HCl (pH 7.4), 1% Triton X-100 (v/v), 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM PMSF] and stirred at 4 °C for 2 h. The solution was centrifuged at $100,000 \times g$ for 30 min, and the supernatant was collected and dialyzed against 20 mM Tris–HCl (pH 7.4) containing 0.1% Triton X-100 (v/v) and 150 mM NaCl [13–15]. The protein content was determined following the method of Lowry et al. [16]. The supernatant was applied

to a SPMG-Sepharose affinity column (1 cm × 6 cm) equilibrated with 20 mM Tris–HCl (pH 7.4) containing 0.1% Triton X-100 (v/v) and 150 mM NaCl. The column was first washed with the loading buffer, and eluted with linear gradient of 0.15–1.5 M NaCl in Tris buffer (pH 7.4) containing 0.1% Triton X-100 at a flow rate of 0.5 mL/min. The elution was monitored at OD 280 nm and estimated with 0.01% Coomassie brilliant blue R-250 (w/v) in ethanol, phosphoric acid and water (1:2:20, v/v). The absorbance was measured at 595 nm with a microplate reader [17,18]. Fractions were pooled and dialyzed against redistilled water followed by concentration.

### 2.5. Electrophoresis and Western blot analysis

The proteins eluted from the column was separated by 15% SDS-PAGE following the method of Laemmli [19]. The gels were stained with 0.2% Coomassie brilliant blue R-250 (w/v) in methanol, acetic acid and water (5:1:5, v/v), and destaining in methanol, acetic acid and water (5:7:88, v/v). Western blotting was conducted following the procedure of Towbin et al. [20]. Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes. After blocking in 5% (w/v) BSA at 4 °C overnight, the membranes were washed with PBS containing 0.05% Tween 20 (v/v), and incubated at 37 °C for 1 h with 1:100 diluted an anti-rat CD4 mAb or the same concentration of nonimmune mouse IgG. The membranes were washed and incubated with a horse anti-mouse IgG conjugated to alkaline phosphatase (1:1000 dilution). The protein bands were visualized by incubating with the developing solution [p-nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate toluidine (BCIP)] at room temperature for 30 min.

### 2.6. SPR assay

Biosensor measurement based on SPR was employed to evaluate interaction between SPMG and hsCD4. In this assay, SPMG was immobilized on the surface of a carboxymethylated dextran sensor chip (CM5) via a biotin–streptavidin capturing procedure. A HBS solution containing hsCD4 at concentrations of 4.3, 8.7, 17.7, 35.5, 71 nM was passed over the sensor chip surface with a flow rate of 5 μL/min, and the changes in mass due to the binding response were measured. Then HBS solution containing coexistence of 71 nM hsCD4 and SPMG at concentrations of 3.125, 12.5, 50, or 200 mg/L was flowed over the sensor chip surface to evaluate the inhibition of free SPMG to the binding of hsCD4 to SPMG immobilized onto the biosensor chip surface.

### 2.7. Statistics

Student's *t*-test and analysis of variance (ANOVA) were performed using Statview. $P < 0.05$ was accepted as significant and $P < 0.01$ was regarded as highly significant.

## 3. Results

### 3.1. SPMG binding to rat and human lymphocytes

Our previous immune studies indicated that SPMG has many immunomodulating activities in vivo and in vitro, including increasing lymphocyte proliferation, enhancing CD69 expression, and increasing the production of intracellular interleukin-2 (IL-2) and interferon-γ (IFN-γ). These observations led us to postulate that there may be SPMG binding sites (receptors) on lymphocyte surface. We first examined the possible binding of SPMG to rat lymphocytes. FCM analysis showed that the fluorescent intensity of lymphocytes in the FITC-SPMG group (100 mg/L) was greater than that of the control group (over two-fold, $P < 0.01$). This finding was confirmed by fluorescence microscopy. As shown in Fig. 2A, thymus lymphocytes of the FITC-SPMG group had bright greenish-yellow grains, while control group exhibited only faint fluorescence. Similar results were obtained both from spleen lymphocytes (Fig. 2B). We further tested the SPMG binding in human lymphocytes. The FITC-SPMG-treated lymphocytes exhibited a much stronger fluorescent intensity (1021.07 ± 8.96 au) than that of control (5.31 ± 0.58 au) ($P < 0.01$), indicating a significant amount of SPMG binding in human lymphocytes (Fig. 2C). Similar binding results were obtained from activated lymphocytes (data not shown).

Next, the effects of different incubation time, concentration, and temperature were investigated. Increases in incubation time (from 30 min to 2 h), concentration (from 25 to 100 mg/L), and temperature (from 4 to 37 °C) resulted in incremental increases in the fluorescent intensity in thymus lymphocytes (from 43.19 ± 1.83 au to 87.61 ± 4.91 au, and from 10.54 ± 1.23 au to 30.32 ± 4.55 au, respectively) (Fig. 3A–C). SPMG binding to lymphocytes reached a plateau at a concentration of 500 mg/L for 2 h at 37 °C (data not shown). Moreover, the binding of SPMG-FITC to lymphocytes was competitively inhibited by unlabeled SPMG ($P < 0.01$). 100 mg/L of unlabeled SPMG exerted a 53.01 ± 2.35% inhibition, while 200 mg/L of free SPMG reached to an 82.90 ± 3.92% inhibition (Fig. 3D). These data indicate that the binding of SPMG to lymphocytes is a rapid, specific, reversible, and saturable process, and imply that SPMG binding to lymphocytes may be a receptor-mediated event.

### 3.2. SPMG binding to several membrane proteins in rat lymphocytes

We choose to use rat lymphocytes to identify and characterize the SPMG binding sites. For this, membrane protein preparation was obtained from spleens and thymus lymphocytes. After solubilization, the preparation was applied to a SPMG-Sepharose affinity column and eluted with a linear gradient of NaCl (0.15–1.5 M) in Tris buffer.

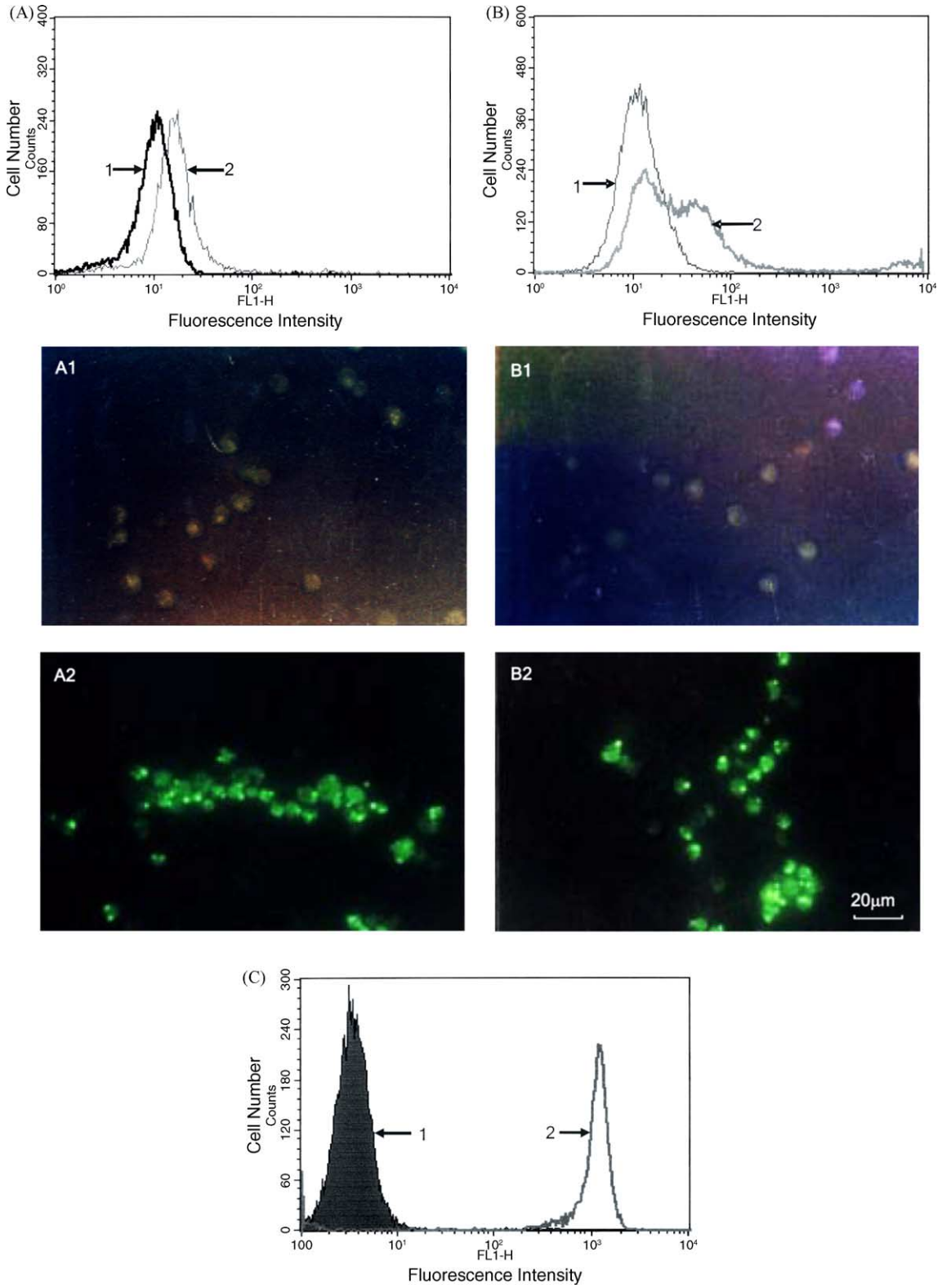


Fig. 2. Binding of SPMG to lymphocytes. Thymocytes (A) and splenic lymphocytes (B) of rat and human lymphocytes (C) were seeded and FITC-labeled SPMG was added at a final concentration of 100 mg/L. After 2 h incubation, cells were harvested, washed, and gated and analyzed by FCM. An aliquot from each sample was spotted on a slide and analyzed and photographed under a fluorescence microscope. The result shown is a representative of three separate experiments. The bright greenish-yellow grains in A2 and B2 indicated the binding of SPMG-FITC to thymic and splenic lymphocytes. A1, B1, C1, control; A2, B2, C2, SPMG-FITC.

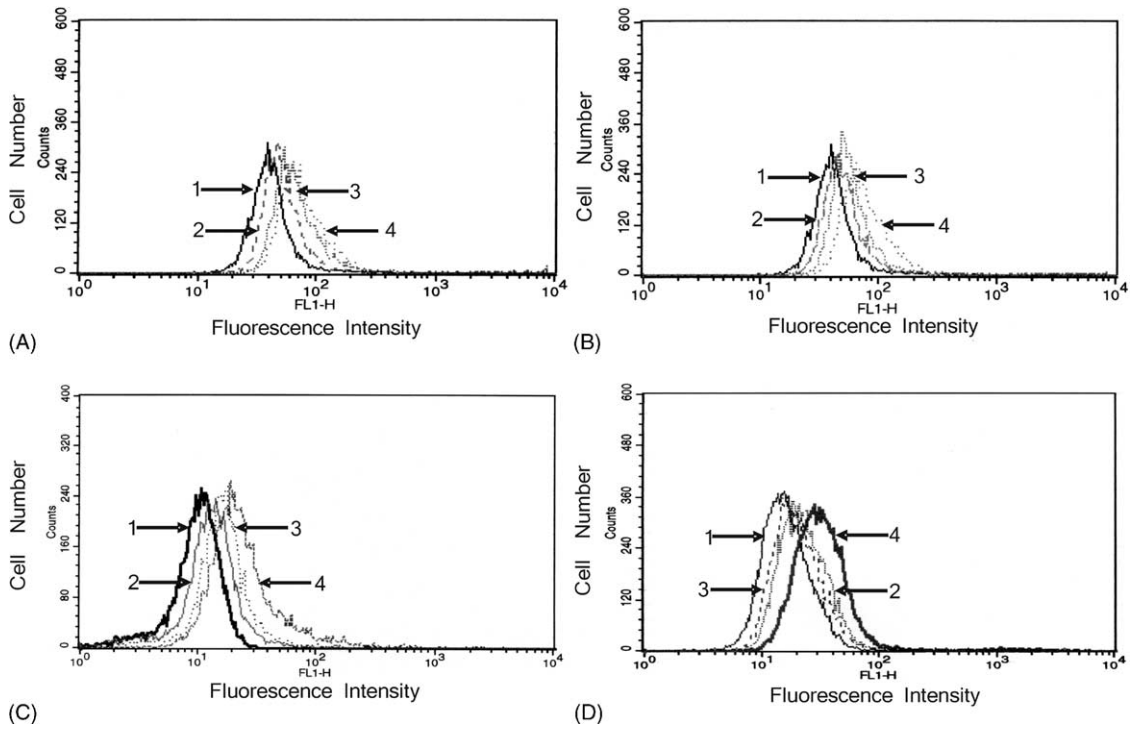


Fig. 3. Characterization of the SPMG binding. Thymocytes were incubated with or without SPMG-FITC at concentrations ranging from 20 to 100 mg/L (A), with periods from 0.5 to 2 h (B), and at 4, 25 or 37°C (C). The reversibility and specificity of the SPMG-FITC binding was examined by incubating thymocytes with SPMG-FITC for 1 h, then adding unlabled SPMG and incubating for another 1 h (D). The standard incubation condition was 100 mg/L SPMG-FITC at 37 °C for 2 h. The bindings were evaluated by FCM and a representative of three separate experiments is shown. A1, control; A2, 0.5 h; A3, 1 h; A4, 2 h. B1, control; B2, 25 mg/L; B3, 50 mg/L; B4, 100 mg/L. C1, control; C2, 4 °C; C3, 25 °C; C4, 37 °C. D1, control; D2, SPMG-FITC + 100 mg/L SPMG; D3, SPMG-FITC + 200 mg/L SPMG; D4, SPMG-FITC.

As the result, a number of proteins were eluted at NaCl concentrations between 0.15 and 0.7 M. At the range from 0.7 to 1.0 M of NaCl, a single symmetric peak was obtained (Fig. 4). Almost no proteins were detected at the concentrations higher than 1.0 M of NaCl (data not shown). The membrane preparations isolated from both thymocytes and splenocytes showed the same elution pattern.

The proteins purified by the SPMG affinity chromatography were subjected to SDS-PAGE under reducing conditions. The gels was visualized by Coomassie staining. Five major bands, ranging from 35 to 64 kDa, were eluted from the thymus membrane fractions at the range of 0.15–0.7 M NaCl. Seven bands with molecular weights ranging from 19 to 44 kDa were detected from the spleen preparation. The fractions eluted from thymocytes with NaCl at concentrations between 0.7 and 1.0 M exhibited two major bands with the apparent molecular masses of 55 and 64 kDa. The spleen fractions had three major bands with molecular sizes of 55, 64 and 75 kDa, respectively (Fig. 5).

### 3.3. SPMG binding to CD4 receptor on rat lymphocytes

We postulated that the 55-kDa protein may be CD4. CD4 is a 55 kDa type I trans-membrane glycoprotein expressed

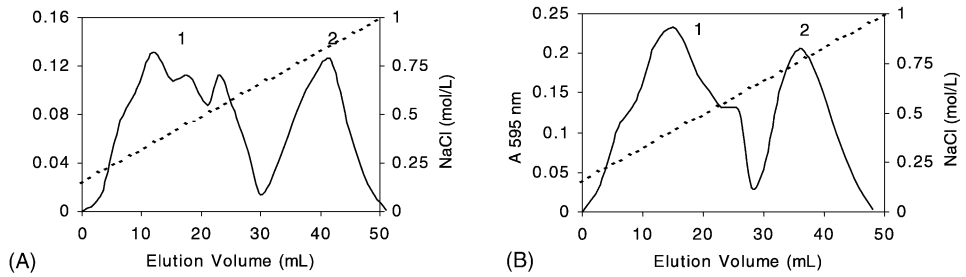


Fig. 4. Purification of lymphocyte membrane proteins binding to SPMG. The solubilized thymus (A) and spleen (B) membrane preparations were applied to a SPMG-Sepharose affinity column and the SPMG-bound proteins were eluted with 0.15–1.5 M linear gradient of NaCl in Tris buffer (pH 7.4) containing 0.1% Triton X-100 (v/v). The eluted proteins were estimated with 0.01% (w/v) Coomassie brilliant blue R-250 in ethanol, phosphoric acid and water (1:2:20, v/v) and the absorbance was measured at 595 nm. Data shown are a representative of three independent experiments.

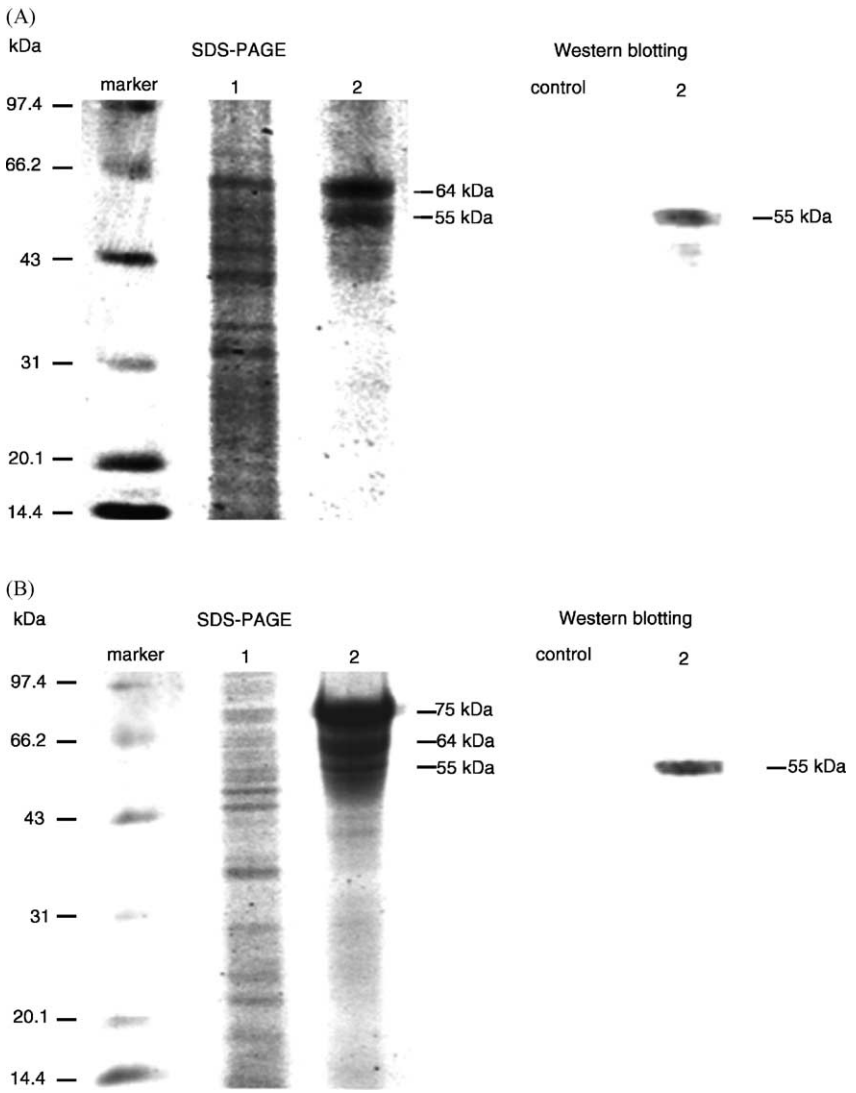


Fig. 5. Characterization of the SPMG binding proteins. Thymus (A) and spleen (B) membrane proteins purified by the SPMG-Sepharose affinity column were seprated by SDS-PAGE (15%) and stained with 0.2% (w/v) Coomassie brilliant blue R-250. Lanes 1 and 2 present fractions 1 and 2 of Fig. 3. Fraction 2 was further subjected to Western blotting analysis using an anti-CD4 mAb. The antibody cross-reacted with the 55 kDa protein (lane 2), while the mouse IgG did not. This is a representative of three independent experiments.

mainly in thymocytes and a subset of mature T lymphocytes [21,22]. Our previous in vivo and in vitro studies have shown SPMG has strong immunopotentiating actions, particularly on T-lymphocytes activation. To test this hypothesis, the fractions obtained by the SPMG affinity column were analyzed by Western blotting using a CD4 mAb. As shown in Fig. 5, the 55-kDa protein was recognized by the CD4 mAb, but not by nonimmune IgG. This indicates that this protein is indeed CD4.

### *3.4. HsCD4 competitively inhibiting SPMG bind to human CD4 of T lymphocytes*

The data above suggested that SPMG interacts with CD4 on the surface of rat thymocytes and T lymphocytes. CD4 in rat and human T cells is closely related to T-cell activation [21]. Human CD4 had been shown to be a major receptor of HIV entry. It specifically binds to gp120 of HIV and plays important roles during HIV infection [23,24]. We, therefore, wished to determine if SPMG can also bind to human CD4. For this, flow cytometric analysis was performed in human peripheral T lymphocytes. The results showed that hsCD4 at concentrations of 25 and 50 mg/L effectively inhibited the binding of FITC-SPMG (1 mg/L) to $CD4^+$ human peripheral T lymphocytes (Fig. 6). The fluorescent intensity was 23.94 ± 3.68 au and 20.08 ± 2.95 au, respectively. These values were significantly lower than the FITC-SPMG alone group (56.96 ± 4.34 au) ($P < 0.01$). These data indicated that the SPMG binds to human CD4 in T lymphocytes.

### *3.5. SPMG binding to hsCD4 molecule in a multivalent manner*

To further investigate the binding kinetics between SPMG and human CD4, the interaction between SPMG

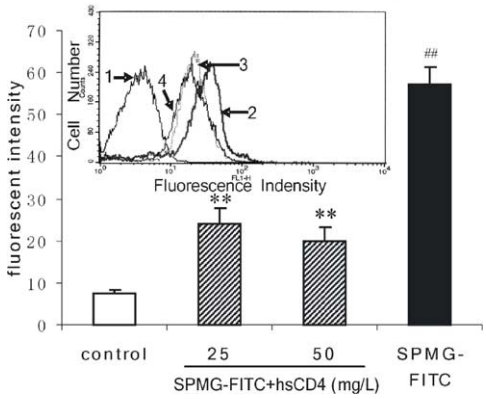


Fig. 6. hsCD4 inhibiting the bind of SPMG to human peripheral blood $CD4^+$ lymphocytes. Lymphocytes obtained from human peripheral blood were incubated with or without 1 mg/L SPMG-FITC in the absence or presence of 25 and 50 mg/L hsCD4 for 2 h, $CD4^+$ lymphocytes were gated and analyzed by FCM. Data shown are a representative of three separate experiments. (1) Control; (2) SPMG-FITC; (3) SPMG-FITC + 25 mg/L hsCD4; (4) SPMG-FITC + 50 mg/L hsCD4. $^{\#\#}P < 0.01$ comparing with control; $^{**}P < 0.01$ comparing with SPMG-FITC group.

and hsCD4 was evaluated by SPR assay. The results showed that SPMG and hsCD4 had a strong association and the binding increases with increasing hsCD4 concentrations (Fig. 7). Kinetic analysis was performed by Biacore evaluation 3.0 software (Amersham Pharmacia). The association constant and dissociation constant were 2.64E + 06 (1/M) and 3.79E − 07 (M) respectively, demonstrating relatively high avidity of SPMG for hsCD4. The stoicheometry is one molecule SPMG binding to six molecules of hsCD4, suggesting that SPMG binds to CD4 in a multivalent fashion. Additionally, as shown in Fig. 8, free SPMG inhibited the binding of hsCD4 to the

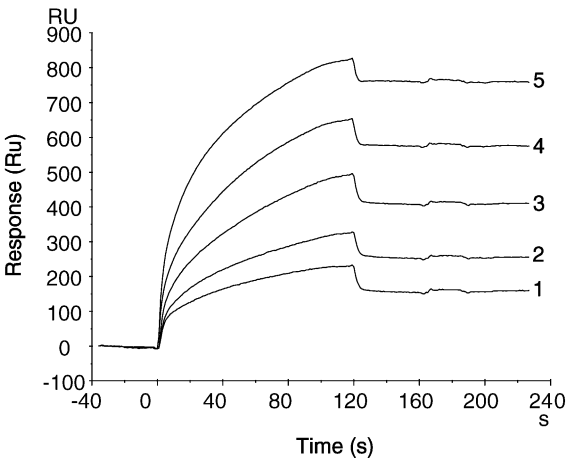


Fig. 7. SPMG binding to hsCD4 in a multivalent manner. The biotin-labeled SPMG was immobilized on the streptavidin-sensor chip surface. Different concentrations of hsCD4 flowed over the sensor chip surface. The concentrations of hsCD4 from bottom to top (1–5) were 4.3, 8.7, 17.7, 35.5 and 71 nM, respectively. *X*-axis stands for flow time and *Y*-axis represents the binding resonance unit. The experiment was carried out at 25 °C, with a flow rate of 5 μL/min. The data shown are a representative of three separate experiments.

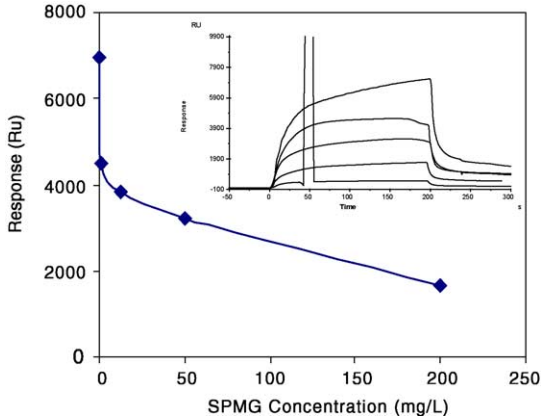


Fig. 8. Inhibitory effect of free SPMG on the binding of hsCD4 to SPMG immobilized onto the biosensor chip surfac. The biotin-labeled SPMG was immobilized on the streptavidin-sensor chip surface. HBS solution containing 71 nM hsCD4 and various concentrations of unlabeled SPMG (3.125, 12.5, 50 or 200 mg/L) was flowed over the sensor chip surface. The inhibition curve is shown and the concentrations of SPMG (3.125, 12.5, 50 or 200 mg/L) are shown in the top right corner (from top to bottom). The experiment was carried out at 25 °C with a flow rate of 5 μL/min. The data shown are a representative of three independent experiments.

SPMG immobilized onto a Biacore sensor chip in a concentration-dependent manner. The $IC_{50}$ was 30 mg/L.

## 4. Discussion

Recent in vivo and in vitro studies have shown that polysaccharides can trigger both cellular and humoral immunoresponse [1–4]. Polysaccharides exert immunomodulating effects by directly binding to the so-called polysaccharide receptors on immunocytes surface, and triggering signal transduction which leads to the immunopotentiating activities [5,9,10]. Our previous studies indicated that SPMG possesses many immunopotentiating activities, including activating immunological cells, macrophages and NK cells, increasing lymphocyte proliferation, and enhancing the intracellular cytokines production [8]. In this study, we show that SPMG binds to lymphocytes in a rapid, specific, reversible, and saturable fashion. Using SPMG affinity chromatography, we have purified the SPMG binders on lymphocytes. SDS-PAGE analysis suggested the presence of two proteins with the size of 55 and 64 kDa in the thymus preparation. Three proteins with the size of 55, 64 and 75 kDa were found in the spleen preparation. We focused on the 55 kDa membrane protein because it has the same molecular mass as CD4 [21,22]. Further Western blotting analysis confirmed that this 55 kDa SPMG binding protein is CD4. SPR analysis indicated that the SPMG binds to hsCD4 in a multivalent manner with specificity. It has been previously shown that the positively charged amino acid side chains within the CD4 molecule serve as an electrostatic docking site for the negatively charged sulfated polysaccharides [25,26]. This polyanion binding site is conserved between

human and rat [27]. It is therefore likely that SPMG binds to CD4 through the conserved polyanion binding. It should be noted that the binding of SPMG to lymphocytes is a rapid, specific, reversible, and saturable process, suggesting that in addition to the electrostatic interaction as a first and essential step, there may be subsequent receptor-mediated binding between SPMG and CD4.

CD4 is expressed predominantly in developing thymocytes and major histocompatibility (MHC) class II restricted mature T lymphocytes. It plays a crucial role during thymocyte differentiation and maturity, T-cell activation, and in the functions of mature T cells [21,22,28]. The extracellular portion of the CD4 molecule contains four functional domains (D1–D4) and one amino terminal signal sequence, while the cytoplasmic portion associates with the Src-related tyrosine protein kinase, p56Lck, which in turn mediates signaling transduction to activate T lymphocyte [28,29]. Our finding on the multivalent binding of SPMG to CD4 might suggest a possible mechanism of cross-linking and oligomerization of CD4 molecules by the SPMG binding, leading to the subsequent T-cell activation by triggering p56Lck-mediated signaling cascade [21,22,29]. Further studies are needed to understand how SPMG acts to modulate the primary immunological abnormality resulting from HIV infection.

CD4 serves as the cell-surface receptor that confers HIV target cell tropism through interacting with the mature virus envelop gp120 [23,24]. The interaction between CD4 and gp120 of HIV triggers conformational changes in gp120, which subsequently leads to virus–cell and cell–cell membrane fusion to form syncytium [24,30,31]. mAb of OKT4A against CD4 and several compounds engaging CD4 have been shown to inhibit HIV infection and syncytium formation [32–36]. Molecules bind to CD4 may favor in docking binding domain of gp120 or interfering with CD4–gp120 binding, leading to a blockage of HIV entry, or inhibiting other post-binding events resulting in membrane fusion and subsequent syncytium formation [37,38]. It is likely that an opportunistic agent that can specifically target host cell CD4 represents a plausible strategy to control HIV infection. In supporting this hypothesis, the multivalent binding of SPMG to CD4 may lead to interference with the association of CD4 with gp120, resulting in the potent anti-HIV activities of SPMG, highlighting potential new opportunities of drug design [7].

To our knowledge, SPMG is the first marine sulfated polysaccharide being considered as anti-AIDS drug candidate. SPMG has successfully passed the Phase I clinical trial, and entered the Phase II trial. In the present study, we have demonstrated for the first time that CD4 molecule is one of the specific binding sites (receptors) of SPMG in lymphocytes. The SPMG binding to CD4 may underlie the immunopotentiation and anti-HIV activities of SPMG in HIV-infected individuals. More studies are needed to identify the 64 and 75 kDa SPMG binding proteins, to determine their functional roles, and to elucidate the underlying signal transduction.

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## References

[1] Hase K, Basnet P, Kadota S, Namba T. Immunostimulating activity of Celosian, an antihepatotoxic polysaccharide isolated from *Celosia argentea*. Planta Med 1997;63(3):216–9.
[2] Liu F, Ooi VEC, Fung MC. Analysis of immunomodulating cytokine mRNAs in the mouse induced by mushroom polysaccharides. Life Sci 1999;64:1005–11.
[3] Wang SY, Hsu ML, Hsu HC, Tzeng CH, Lee SS, Shiao MS, et al. The anti-tumor effect of *Ganoderma lucidum* is mediated by cytokines released from activated macrophages and T lymphocytes. Int J Cancer 1997;70(6):699–705.
[4] Oka M, Hazama S, Suzuki M, Wang F, Wadamori K, Iizuka N, et al. In vitro and in vivo analysis of human leukocyte binding by the antitumor polysaccharide, Lentinan. Int J Immunopharmacol 1996;18(3):211–6.
[5] Honda S, Campbell JJ, Andrew DP, Engelhardt B, Butcher BA, Warnock RA, et al. Ligand-induced adhesion to activated endothelium and to vascular cell adhesion molecule-1 in lymphocytes transfected with the *N*-formyl peptide receptor. J Immunol 1994;152(8):4026–35.
[6] Xin XL, Geng MY, Guan HS, Li ZL. Study on the mechanism of inhibitory action of 911 on replication of HIV-1 in vitro. Chinese J Marine Drugs 2000;19:15–8.
[7] Geng MY, Li FC, Xin XL, Li J, Yan ZW, Guan HS. The potential molecular targets of marine sulfated polymannuroguluronate interfering with HIV-1 entry. Interaction between SPMG and HIV-1 rgp120 and CD4 molecule. Antivir Res 2003;59(2):127–35.
[8] Miao BC, Geng MY, Li J, Li FC, Guan HS, Xia W. Study of immunopotentiating effects of marine sulfated polysaccharide of 911. Chinese J Marine Drugs 2002;5(21):1–4.
[9] Liang H, Reich CF, Pisetsky DS, Lipsky PE. The role of cell surface receptors in the activation of human B cells by phosphorothioate oligonucleotides. J Immunol 2000;165:1438–45.
[10] Willment JA, Gordon S, Brown GD. Characterization of the human β-glucan receptor and its alternatively spliced isoforms. J Biol Chem 2001;276(47):43818–23.
[11] Chen MZ, Wei W, Zhang H, Liang JS. The experimental methods of immunosuppressive agents and immnostimulants. In: Xu SY, Bian YN, Chen X, editors. The experimental methodology of pharmacology, 2nd ed. Beijing: People's Medical Publishing House; 1994. p. 1208–45.
[12] Konrad Z, Eichler J. Protein glycosylation in *Haloferax volcanii*: partial characterization of a 98-kDa glycoprotein. FEMS Microbiol Lett 2002;209:197–202.
[13] Hao HN, Lyman WD. HIV infection of fetal human astrocytes:the potential role of a receptor-mediated endocytic pathway. Brain Res 1999;823:24–32.

[14] Geetha C, Deshpande V. Purification and characterization of fish liver ferritins. Comp Biochem Phys B 1999;123:285–94.
[15] Maeda S, Kashiwabara Y. Purification and properties of a membrane-bound phospholipase B from *Mycobacterium lepraemurium*. Biochim Biophys Acta 1996;1303:31–8.
[16] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with folin phenol reagent. J Biol Chem 1951;193:265–75.
[17] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 1976;72:248–54.
[18] Krauspe R, Scheer A. Coomassie brilliant blue G-250 dye-binding technique for determination of autolytic protein breakdown in *Euglena gracilis* and comparison to other methods of autolysis measurement. Anal Biochem 1986;153(2):242–50.
[19] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage, T4. Nature 1970;227:280–5.
[20] Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. P Natl Acad Sci USA 1979;76:4350–4.
[21] Veillette A, Bookman MA, Horak EM, Samelson LE, Bolen JB. Signal transduction through the CD4 receptor involves the activation of the internal membrane tyrosine-protein kinase p56lck. Nature 1989;338(6212):257–9.
[22] Foti M, Phelouzat MA, Holm A, Rasmusson BJ, Carpentier JL. p56Lck anchors CD4 to distinct microdomains on microvilli. Proc Natl Acad Sci USA 2002;99(4):2008–13.
[23] Moir S, Boissinot M, Tremblay M, Poulin L. CD4 deletion mutants evaluated for human immunodeficiency virus type 1 infectivity in a highly efficient system of expression and detection based on LTR-dependent reporter gene activation. J Virol Methods 1997;65(2):209–17.
[24] Sattentau QJ, Moore JP. The role of CD4 in HIV binding and entry. Philos Trans R Soc Lond B Biol Sci 1993;342(1299):59–66.
[25] Sze'kely Z, Fa'bia'n P, Torday LL, Michejda CJ, Aszalo's A. Binding between the CD4 receptor and polysulfonated azo-dyes. An exploratory theoretical study on action-mechanism. J Mol Struct 1998;423:153–9.
[26] Dougal JS, Klatzmann DR, Maddon PJ. CD4–gp120 interactions. Curr Opin Immunol 1991;3(4):552–8.
[27] Parish CR, Warren HS. Conservation of a polyanion binding site in mammalian and avian CD4. Immunol 1991;74(2):191–6.
[28] Bowers K, Pitcher C, Marsh M. CD4: a co-receptor in the immune response and HIV infection. Int J Biochem Cell Biol 1997;29(6): 871–5.
[29] Wu H, Kwong PD, Hendrickson WA. Dimeric association and segmental variability in the structure of human CD4. Nature 1997;387(6632):527–30.
[30] Sattentau QJ, Moore JP. Conformational changes induced in the human immunodeficiency virus envelope glycoprotein by soluble CD4 binding. J Exp Med 1991;174(2):407–15.
[31] Moore JP, Doms RW. The entry of entry inhibitors: a fusion of science and medicine. Proc Natl Acad Sci USA 2003;100(19):10598–602.
[32] Burkly L, Olson D, Shapiro R, Winkler G, Rosa J, Thomas D, et al. Inhibition of infection by novel CD4 domain 2-specific monoclonal antibodies. J Immunol 1992;149:1779–87.
[33] Truneh A, Buck D, Cassatt DR, Juszczak R, Kassis S, Ryu SE, et al. A region in domain 1 of CD4 distinct from the primary gp120 binding site is involved in HIV infection andvirus-mediated fusion. J Biol Chem 1991;266:5942–8.
[34] Hasunuma T, Tsubota H, Watanabe M, Chen Z, Lord C, Burkly L, et al. Regions of the CD4 molecule not involved invirus binding or syncytia formation are required for HIV-1 infection of lymphocytes. J Immunol 1992;148:1841–6.
[35] Nagashima KA, Thompson DA, Rosenfield SI, Maddon PJ, Dragic T, Olson WC. Human immunodeficiency virus type 1 entry inhibitors PRO 542 and T-20 are potently synergistic in blocking virus–cell and cell–cell fusion. J Infect Dis 2001;183(7):1121–5.
[36] Rusconi S, Moonis M, Merrill DP, Pallai PV, Neidhardt EA, Singh SK, et al. Naphthalene sulfonate polymers with CD4-blocking and anti-human immunodeficiency virus type 1 activities. Antimicrob Agents Chemother 1996;40(1):234–6.
[37] Lederman S, Gulick R, Chess L. Dextran sulfate and heparin interact with CD4 molecules to inhibit the binding of coat protein (gp120) of HIV. J Immunol 1989;143(4):1149–54.
[38] Watson K, Gooderham NJ, Davies DS, Edwards RJ. Interaction of the transactivating protein HIV-1 tat with sulphated polysaccharides. Biochem Pharmacol 1999;57:775–83.