ART & TECHNOLOGY PUBLISHING PTE. LTD.

Gene Science and Engineering

2021 Volume 3, Issue 1

ISSN: 2529-7651

Marine Natural Products and Immunoactive Substances, Basic Research of α-Galactosylceramides

Takenori Natori*

Faculty of Pharmaceutical Sciences, Teikyo Heisei University, Nakano-ku, Tokyo 164-0001, Japan

***Corresponding author:** Takenori Natiro, t.natori@thu.ac.jp

Abstract

Marine organisms produce second metabolites with unique chemical structures because of their complex symbiotic systems different from that of land organisms. Unique glycosphingolipids with α-galactosylceramide structure, agelasphins, were obtained from the Okinawan marine sponge *Agelas mauritianus* by the *in vivo* antitumor substance screening system. Agelasphins and the developmental candidate, KRN7000 (α-GalCer), markedly showed antitumor activity against tumor-bearing mice by activating the host's immune system. The discovery of agelasphins and the development of KRN7000 revealed the detail of natural killer T cell functions. Unfortunately, the development of KRN7000 intended to be an antitumor drug by systemic injection was abandoned because of several limitations. Now it is strongly expected that agelasphin or its derivative to be developed as a novel immunomodulator by conquering the limitations.

Keywords

Agelasphin
α-Galactosylceramide
α-GalCer
KRN7000
NKT cell

1. Introduction

Marine organisms are known to produce secondary metabolites with unique structures due to complex symbiotic mechanisms that differ from those of terrestrial and freshwater organisms, and many attempts have been made to utilize them in drug discovery.

Natural killer T cells (NKT cells) are immunocompetent cells that bridge innate and acquired immunity and are unique in their ability to induce the Type 1 T-helper (Th1) cytokine production as well as the Type 2 T-helper (Th2) cytokine induction. Both of these immune inductions are triggered by the presentation of sphingoglycolipids (SGLs), which have ceramide in their lipid moiety and sugar chains attached to them in a specific conformation, to CD1d molecules on dendritic cells, which are recognized and activated by T-cell antigen receptor (TCR) expressing invariant NKT (iNKT) cells.

Among these SGLs, α-GalCer (KRN7000), also known as agelasphin (AGL), is a very potent NKT cell activator derived from Okinawan sponges *Agelas mauritianus*, which was discovered by a team from

Kirin Brewery Company through drug discovery screening from marine organisms. AGLs have neutral sugars in their sugar chains, but SGLs with acidic sugars were subsequently isolated from gram-negative bacteria of the genus *Sphingomonas* and shown to exhibit similar activity.

The 'true producers' of AGLs are likely microorganisms living in a sponge symbiosis, and some SGLs have been reported to be involved in bacterial-host interactions by constituting lipid bilayer rafts, and the significance of their presence in the host is also of interest. In recent years, chemical synthesis of various derivatives of AGLs is underway, and it is expected to contribute to the treatment and prevention of immune disorders, cancer, and infectious diseases by freely controlling their unique actions.

2. Discovery of agelasphins

At its peak in the 1980s, pharmaceutical companies around the world were conducting drug discovery research using natural product chemistry based on the study of secondary metabolites of microorganisms such as actinomycetes. In addition, different screening methods were used, including common methods of searching for anticancer drugs based on cytotoxicity, leading to the discovery of substances with a variety of unique structures and physiological activities.

On the other hand, it has long been suggested that marine organisms may produce different types of metabolites from terrestrial organisms. In particular, studies of organisms in the reef areas where they can be collected by scuba diving have revealed their complex symbioses. In areas where sunlight reaches the organisms, complex primary and secondary symbioses are formed, with photosynthetic cyanobacteria at the top, and the genomes of symbionts and host cells are thought to integrate and change into an inter-organelle metabolic product transport system as organelle linkages progress and intracellular symbioses are established. This complex symbiotic relationship enables the production of a variety of metabolites, and it is thought that many metabolites of marine organisms do not fit the metabolic model of terrestrial organisms, which has attracted natural product chemists to the ocean [1,2]. This has led to the discovery of various metabolites from marine organisms, and further research into the true producers of these metabolites. Unfortunately, due to several challenges, only a few compounds have reached the stage of clinical use as drugs, including plitidepsin (also known as dehydrodidemnin B, Aplidin®) and trabectedin (also known as ecteinascidin-743, Yondelis®) being the main drugs that have antitumor activity (**Figure 1**).

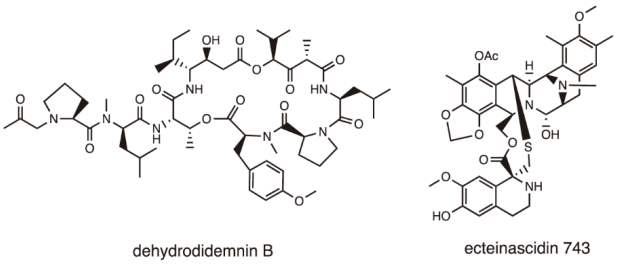

Figure 1. Examples of drugs derived from marine organisms

The trend has been followed to conduct a screening of antitumor substances at the (then) Kirin Brewery Company's Pharmaceutical Discovery Research Institute. Initially, extracts of marine organisms were prepared and screening was carried out relying on clear indicators such as cytotoxicity, but it was difficult to find the expected candidate substances as drugs. However, it was then decided to determine the overall potential of the extracts by injecting them directly into the abdominal cavity of C57BL/6 mice transplanted with melanoma from B16 mice and examining their life-prolonging effects. The screening process changed when the technician in charge of animal care reported that the mice injected with the extract had a very good hair coat for a long time, although the life-prolonging effect was not so great. Normally, black mice transplanted with cancer cells would have extremely bad hair, but the mice treated with this extract kept their hair glossy right up to the time of death. Until then, antitumor agents had been associated with strong toxicity and preferential killing of fast-growing tumor cells, but the administration of

this sponge extract strengthened the immune system of the mice themselves *in vivo*, and it was confirmed to possibly eliminate tumors. Subsequently, a search was conducted for active substances using this 'hair gloss' as an indicator, resulting in the isolation and structural analysis of a series of new α-galactosylceramides from the Okinawan sponge *Agelas mauritianus*, and naming them agelasphins. The structure of AGL-9b, the most abundant agelasphin, is shown in **Figure 2**. Although not discussed in detail in this paper, it has not been clarified whether the 'true producers' of agelasphins are the sponges or the symbiotic microorganisms, due to the aforementioned complexity of symbiotic relationships in natural products derived from marine organisms.

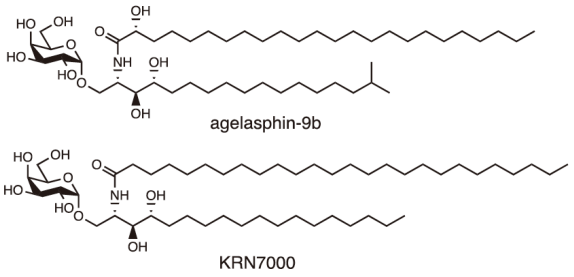

Figure 2. Chemical structures of agelasphins (agelasphin-9b) and KRN7000.

3. Agerasphin-related compounds and derivative synthesis

Agerasphene is a sphingolipid composed of ceramide, which consists of α-hydroxy fatty acids and phytosphingosine, and D-galactose. The most important structural feature is the α-coordination of galactose to ceramide. Although a series of monoglycosylceramides, called cerebrosides, have been known for a long time and have been chemically synthesized, there have been no reports on cerebrosides with α-coordinated sugars and their biological activities were unknown.

Later, however, agelasphin and a group of compounds with higher sugar linkages were isolated from other sponges of the genera *Agelas* and *Stylissa*, indicating that agelasphin is not necessarily a rare compound but is relatively widely distributed in sponges. However, it had never been reported that sphingoglycolipids showed such a strong antitumor effect, and pharmacological effects of unknown impurities such as lipopolysaccharides, which remained in the purified agelasphin in very small amounts, were also suspected, and even a limulus test of the purified product was conducted. However, in the end, a stereospecific total synthesis of AGL-9a and AGL-9b, which have simple structures among the agelasphins, was achieved in 13 steps, and these compounds showed physicochemical properties identical to those of naturally occurring compounds, as well as equivalent physiological activity. The results confirm that agelasphin is the active body of the antitumor activity exhibited by the sponge extract, and its structure, including the absolute stereo configuration, has been established.

Furthermore, more than 100 derivatives were synthesized to find low-cost synthesizable derivatives for pharmaceutical development. The derivatives were vigorously investigated and synthesized in many aspects, including the type and number of sugars, the mode of bonding, the stereochemistry of the ceramide moiety, and the number of hydroxyl groups. The original structures were composed of three parts, a highly hydroxylated sugar, a long-chain base, and a fatty acid, which were linked by glycosidic and amidic bonds, However, the number of modification sites was not easy to optimize because they were too numerous. In the end, physiological activity was prioritized and a compound named KRN7000 was selected as a candidate for development based on its antitumor activity in mice and its ability to induce differentiation of human cord blood-derived cells, but this led to several issues as described in the next section (**Figure 2**) [3,4].

4. Elucidation of agelasphin's mechanism of action

Agelasphin showed a remarkable life-prolonging effect in mice with carcinoma *in situ*, despite showing little *in vitro* cytotoxicity or *in vivo* toxicity to animals, suggesting that it acts on some host immune systems.

The effects on T cells and NKT cells, which were known at the time to be responsible for antitumor properties, were investigated. As a result, it was confirmed that induction of splenocyte proliferation occurred by activating splenocytes both *in vivo* and *in vitro*, and it was of interest what kind of cells in splenocytes contributed to the antitumor effect of agelasphin.

In this context, KRN7000, which was successfully prepared in large quantities as a candidate substance for development, was made available to collaborators around the world, and energetic research was conducted to elucidate its mechanism of action and its applications. As a result, it was found that dendritic cells (DCs), which are antigen-presenting, activate immune cells via their CD1d molecules, and NKT cells are the cells with the greatest contribution to antigen presentation. At the time, NKT cells were a cell lineage whose activation mechanism and other aspects remained unclear, but the discovery that KRN7000 was a ligand that activated NKT cells extremely strongly via DCs accelerated NKT cell research [5].

Today, it is widely accepted that activation of NKT cells by KRN7000, sphingolipids with similar α-glycosylceramide structures or their analogs, enhances interferon- γ (IFN-γ) and interleukin-12 (IL-12) secretion, which in turn induces activation of Th1 cells. The activation of Th1 cells by IFN-γ and IL-12 secretion is induced by the activation of NKT cells with glycosphingolipids or their analogs, and the activation of Th2 cells is also possible by stimulating IL-4 secretion at the same time. In the future, the development of drugs and vaccine adjuvants for antitumor and anti-infectious diseases by enhancing the immunostimulatory effects of NKT cells using their unique properties, or drugs and therapies for antiallergy, anti-autoimmune diseases and anti-graft versus host disease (GvHD) during organ transplantation based on their immunosuppressive effects, is expected.

5. Formulation and early clinical trials of KRN7000

In 1999, a clinical trial of KRN7000 was planned in the hope that systemic intravenous administration of KRN7000 would have an antitumor effect. The initial step was to synthesize the required amount of the investigational drug substance. Although the drug substance was prepared in accordance with the GMP standards for investigational new drugs at the time, as aforementioned, the extremely long number of steps involved made the process extremely difficult.

Furthermore, for intravenous administration, solubilization in a solvent that can be administered as an injectable drug is a matter of course, but the solubility of KRN7000 in water and alcohol was pessimistically low. In animal studies, it is common practice to dissolve KRN7000 in an organic solvent such as dimethylsulfoxide (DMSO) and then dilute it in a buffer, but in clinical practice, such a technique is naturally not available, so the formulation of the drug was very difficult.

Eventually, a formulation was developed in which the KRN7000 was dissolved using several surfactants and excipients and lyophilized: a small vial containing 200 μg of KRN7000 in one bottle, which was easily dissolved by adding 1 mL of distilled water for injection and stirring (**Figure 3**). This formulation is still used to dissolve KRN7000, which is sold as

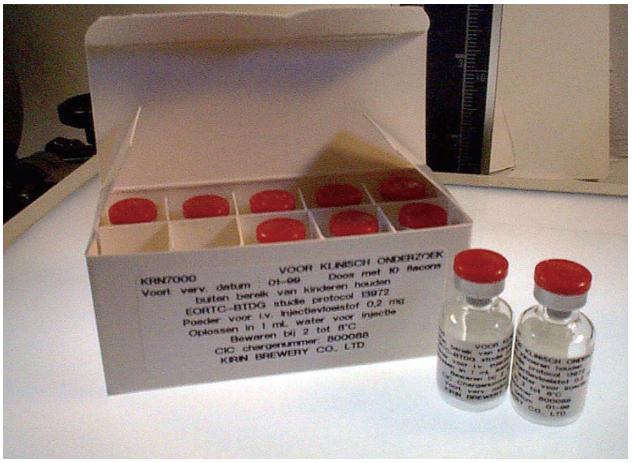

Figure 3. The intravenous formulation of KRN7000 developed for clinical trials

a reagent, and the development of this formulation was a breakthrough in the conduct of clinical trials of KRN7000. There is no doubt that the efforts and results of researchers in the mass synthesis and formulation of many drugs have made a significant contribution to drug treatment and should be more widely known, but it is a pity that these techniques are not widely publicized due to the possibilities of being sensory and artisanal.

The initial clinical trial was planned in the Netherlands, and as it was a first-in-man study, a very low dose and careful administration schedule was set up, with weekly intravenous infusions given once a week for three weeks followed by a two-week rest cycle, for a maximum of six courses. A maximum of six courses were administered to patients with solid tumors. The maximum dose of 4,800 μg/m^2 was increased without any safety problems and the high tolerability of KRN7000 was confirmed. Although some patients showed no changes (NC) or partial response (PR) in some cancer types, the overall efficacy of KRN7000 was not as significant as that shown in mice [6]. To our great dismay, even those patients who had responded well to the first dose of KRN7000, with the secretion of various cytokines, did not respond at all to the second and subsequent doses. This was thought to be due to so-called anergy (immune refractoriness) in the immune response, but the mechanism of this phenomenon is still unclear. Clinical trials were planned and conducted in healthy subjects and hepatitis patients overseas, all to bring KRN7000 to the market, but it was concluded that the effect of systemic administration of KRN7000 in humans was limited and its development was halted.

Typically, scientific interests in KRN7000 and agelasphin would have been lost with the decision to halt its development, but up to date, KRN7000 is widely marketed as a reagent for NKT cell research, as it is recognized as the 'strongest' ligand for activating NKT cells. It has also been the subject of development as a reagent for NKT cell research, as well as derivatization, DDS (drug delivery), and as an *ex vivo* cell activator in cell transfer therapies. The fact that such activity still exists at the end of 2018, nearly 30 years after the discovery of agelasphin, is surprising and illustrates how attractive and promising the development of NKT cell-mediated therapies for various diseases is.

6. Conclusion

Some of the shortcomings of KRN7000 as a pharmaceutical product that were revealed in early clinical trials have not been overcome to date. However, the detailed functions revealed by KRN7000 have not diminished the appeal of NKT cells and the goal of developing therapies for immune-related diseases using these cells but rather increased their appeal.

It is strongly hoped that the entry of many scientists into this field will stimulate NKT cell-related research and that someday medical treatment using NKT cells will be established in some form. At that time, agelasphin derived from marine organisms will come back into the limelight, and meanwhile, the field of marine natural product chemistry will also become more active.

Disclosure statement

The author declares no conflict of interest.

References

[1] Shiraiwa Y, 2015, Photosynthetic Biomass Production by Marine Microalgae and Its Impact on Global Environment. Chemical Times, 238: 2–9.

[2] Matsunaga K, Takeyama H, Yokouchi Y, 2005. Prospects for Marine Genome Research. Journal of the Japanese Society of Seawater Science, 59: 4–11.

[3] Uchimura A, Shimizu T, Morita M, et al., 1997, Immunostimulatory Activities of Monoglycosylated α-D-Pyranosylceramides. Bioorg Med Chem, 5: 2245–2249.

[4] Tashiro T, Mori K, 2010, Fifteen Years Since the Development of KRN7000 – Structure-Activity Relationship Studies on Novel Glycosphingolipids which Stimulate Natural Killer T Cells. Trends Glycosci Glycotechnol, 22: 280–295.

[5] Koezuka Y, Motoki K, Sakai T, et al., 1999, Antitumor Activity of KRN7000 (α-Galactosylceramide) and Its Novel Immunostimulatory Mechanism. Recent Res Devel Cancer, 1: 341–354.

[6] Giaccone G, Punt CJ, Ando Y, et al., 2002, A Phase I Study of the Natural Killer T-Cell Ligand Alpha-Galactosylceramide (KRN7000) in Patients with Solid Tumors. Clin Cancer Res, 8: 3702–3709.