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Neutralizing snake venoms: Costa Rica



GENERAL INFORMATION

◆ **Implementing institution**

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◆ **Implementation period**

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◆ **Costs**

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SUMMARY

Envenomations due to snakebites constitute a significant public health problem in tropical and subtropical countries. A dramatic consequence of snakebites is local muscle destruction (myonecrosis) caused by a group of toxins that affect skeletal muscle cells (myotoxins). This project investigated the neutralizing properties of fucoidan, a negatively charged polysaccharide extracted from the brown seaweed *Fucus vesiculosus*, towards myotoxins isolated from different snake venoms. This compound was selected on the basis of recent advances in the knowledge of the molecular structure of the toxins and their mode of action. Results showed that fucoidan forms complexes with the myotoxins and inactivates their muscle-damaging activity. Preclinical assessment of the neutralizing ability of fucoidan in experimentally envenomed mice demonstrated that the rapid local administration of fucoidan can prevent up to 50 per cent of the muscle damage typically induced by snake venom. These investigations highlight the potential of an inexpensive natural product, fucoidan, or possible derivatives of it, as a complementary treatment to serotherapy against the muscle-damaging activity of snake venoms.

BACKGROUND AND JUSTIFICATION

Each year, more than 50,000 people are killed by bites from poisonous snakes, mostly in tropical and subtropical coun-

tries. Many more people survive being bitten but suffer serious and often permanent injuries as a result of the bite. Envenomations due to snakebites, therefore, are an important public health problem and have a significant socioeconomic impact.

Among the most dramatic consequences of snakebites is local damage to muscle tissue, or myonecrosis. In envenomations by crotaline snakes (which include rattlesnakes and other pit vipers), this effect is mediated mainly by myotoxins, a group of toxins that attack skeletal muscle cells.

Typically, poisonous snakebites are treated by administering antibody preparations (known as antivenoms) that bind to the various venom toxins and block their deleterious activities. These antivenoms are usually obtained from the sera of immunized animals such as horses and sheep. Often, however, antivenoms do not contain sufficiently high levels of neutralizing antibodies to be effective against the myotoxins. In addition, antibodies are large molecules that diffuse slowly into the patients' tissues, which contrasts with the rapid action and spread of the toxins. The effectiveness of conventional serotherapy with antivenoms, therefore, has important limitations.

This problem has motivated the search for alternative neutralizing molecules that could complement and enhance the conventional antivenom treatment. The long-term goal of such studies is to provide new pharmaceutical tools to prevent, as much as possible, the

development of irreversible lesions in patients bitten by poisonous snakes that may leave them with permanent tissue loss and, in severe cases, disability (fig. 1).



Figure 1 | Severe local tissue damage in the hand after a bite from a species of *Bothrops* snake.

DESCRIPTION

Viperid snakes (including the subfamilies Crotalinae and Viperinae) are widely distributed in both the Old World and the New World. The group comprises a large number of species that are responsible for the majority of snakebite envenomations. Their venoms induce conspicuous local tissue damage, and myonecrosis is a major clinical concern. This effect is caused mainly by a group of proteins with phospholipase A₂ (PLA₂) structure, the myotoxins (fig. 2). Within the last two decades, a number of myotoxic PLA₂s have been isolated from the venoms of crotaline species in the genera *Agkistrodon*, *Atropoides*, *Bothriechis*, *Bothrops*,

Calloselasma, *Cerrophidion*, *Crotalus*, *Porthidium* and *Trimeresurus*.

For several years, the Instituto Clodomiro Picado has focused on the biochemical and biological characterization of these toxins, with the aim of providing a more rational basis for the development of alternative, efficient inhibitors that may be used as a complementary treatment against tissue damage induced by snake venoms. Recent advances in the basic knowledge of the structural and functional properties of the myotoxic PLA₂s have shown that a highly cationic (positively charged) and hydrophobic region, located at a particular point in the myotoxin protein — the C-terminal loop — is crucial for the expression of their toxic activity. Theoretically, therefore, highly anionic (or negatively charged) molecules, if they are capable of binding to and blocking the active toxin region, are good candidates for novel neutralizing compounds.



Figure 2 | Three-dimensional crystal structure of *Bothrops asper* myotoxin II.

First, a myotoxic PLA₂ was purified from the venomous *Bothrops asper*. Then, to minimize the use of experimental animals, an *in vitro* assay involving the use of cultured skeletal muscle cells as targets was developed to test the activity of the *B. asper* PLA₂. Potential inhibitors were pre-incubated with the toxin before the mixture was added to the cells. Cytotoxic activity was quantified by determining the release of the enzyme lactate dehydrogenase (LDH) into the culture fluid. A positive neutralization was indicated if the release of LDH was prevented (compared to control reactions containing no inhibitor compound).

With this assay, it was possible to screen a number of possible inhibitors and select only the most promising for *in vivo* testing in mice. Among these was fucoidan, a sulphated polyanionic polysaccharide extracted from the brown seaweed *Fucus vesiculosus*. Under such assay conditions, fucoidan was able to neutralize the *B. asper* toxin. Indeed, neutralization of the myotoxins by fucoidan occurred very rapidly, as exemplified by the results obtained with *Bothrops asper* myotoxin, which gave complete neutralization of toxic activity within the first 5 minutes of pre-incubation (fig. 3).

Neutralization was similarly achieved when a panel of myotoxins, isolated from a variety of crotaline snake species, were tested.

After the positive *in vitro* results obtained with fucoidan using purified myotoxins, preclinical *in vivo* tests of its

ability to neutralize the myotoxic activity of a range of PLA₂s as well as crude *B. asper* venom were carried out. The purified myotoxins or crude venom were injected into mice either alone or after pre-incubation with fucoidan. After three hours, skeletal muscle damage was estimated by determining the creatine kinase (an indicator of muscle cell necrosis) level in the blood.

The results demonstrated that fucoidan was also capable of neutralizing the muscle-damaging activity of the myotoxins *in vivo*, with creatine kinase levels generally less than 25 per cent of those found when the toxins were not pre-incubated with fucoidan (fig. 4). This indicates a good correlation between results obtained in the *in vitro* cytotoxicity model used as a screening procedure and the *in vivo* myotoxicity. The myotoxic action of the crude venom of *B. asper*

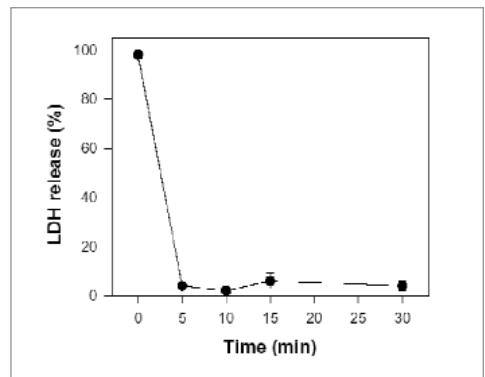


Figure 3 | Time-course reaction of the inhibition of the cytotoxic activity of *B. asper* myotoxin II by fucoidan *in vitro*. Cytotoxicity was determined by the release of lactate dehydrogenase (LDH) from a culture of muscle cells.

was also abolished by pre-incubation with fucoidan, demonstrating that its effect is mainly mediated by the myotoxic PLA₂s studied as isolated toxins.

Finally, the efficiency of fucoidan in preventing muscle damage when rapidly administered to experimentally envenomed mice was tested. The results confirm that, if immediately injected *in situ*, fucoidan prevented up to 50 per cent of the myonecrosis that would develop after the injection of *B. asper* venom.

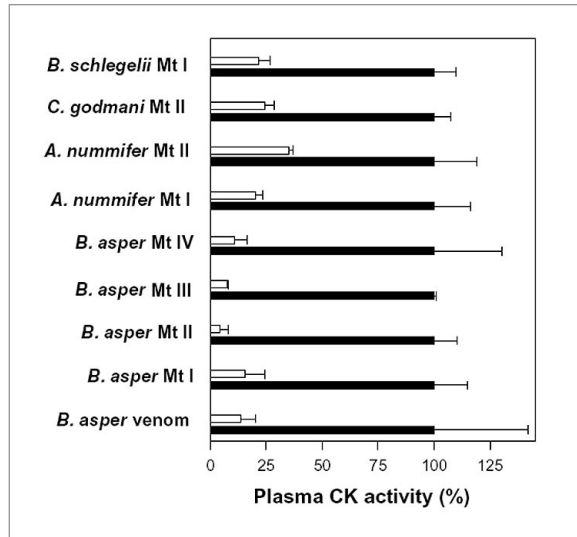
PATENTING AND COMMERCIALIZATION

Fucoidan is a readily available, low-cost natural polysaccharide extracted from

brown seaweed, which should be a renewable resource in many geographical areas. Its production has been commercialized in several Asian countries where it is sold as a kind of nutritional supplement with medicine-like properties.

From an economic point of view, it is interesting to note that conventional antidotes against snake venoms (antivenoms) are considered almost as “orphan” drug products and are not a priority for large pharmaceutical companies. Indeed, several cases exist where private producers have decided to close antivenom preparation facilities owing to a lack of profitability, leaving entire geographical areas unprotected (Africa is an example). In some countries, antivenom production is subsidized by governments or performed by public, non-profit institutions.

Figure 4 | Inhibition of the *in vivo* myotoxic activity of PLA₂s from different species of snake and crude *B. asper* venom by fucoidan. Myotoxins or crude *B. asper* venom was injected intramuscularly either alone (filled bars) or pre-incubated with fucoidan (empty bars). Blood creatine kinase (CK) levels were determined after three hours. Values are expressed as a percentage of the CK activity resulting from the injection of each toxin (or venom) alone. The blood CK values of mice receiving a saline injection alone were subtracted in all cases. Each bar represents the mean ± the standard deviation obtained from four mice.



PARTNERSHIPS

This project has largely been carried out independently of collaborations with other groups, although Mats Wahlgren (Karolinska Institute, Sweden) first introduced the study of fucoidan to scientists at the Instituto Clodomiro Picado.

REPLICABILITY

This research experience, which to date has reached only the preclinical level, may become of interest and use in other tropical regions of the world where bites from crotaline snakes are an everyday hazard. Such accidents are numerous on the American continent, especially in Latin America, as well as in Asia. The myotoxins studied are sufficiently conserved in different snake species to warrant neutralization, as suggested by the results of this project. If fucoidan (or derivatives of it with improved neutralizing efficiency) becomes useful as a complementary treatment in snakebites, this could benefit a significant number of patients.

POLICY IMPLICATIONS

For maximum effect, inhibitors such as fucoidan, or a derivative of it, must be administered immediately after a snakebite. This would require the production of a simple injection device containing the inhibitor that could be distributed to people in high-risk areas, such as farmers or other groups frequently

exposed to the natural habitat of snakes. A public policy to support distribution of the eventual product through rural health care providers would also be relevant.

LESSONS LEARNED

An important component of the success of this project was the ability to combine basic research work with applied studies. By starting with a knowledge of the structural and biological properties of crotaline snake toxins instead of randomly screening a large number of natural compounds, Institute scientists significantly increased the probability of finding a natural inhibitor. In this regard, it would seem very important to promote not only applied research in developing countries but also parallel efforts focusing on basic aspects of scientific problems, with an appropriate balance between the two. The success of the project also stems from the fact that the research team had been working on subjects that have become established lines of research in the Instituto Clodomiro Picado for a number of years. Continuity in the subject allowed the team to gather useful experience and knowledge strength and to combine in-depth questions with parallel horizontal explorations.

A common obstacle in the local scientific and technological environment is the scarcity of national public or private funding for research. In addition, it is difficult to obtain supplies rapidly and efficiently. Another problem concerns limited access to scientific journals, which is

compounded by the decreasing number of subscriptions in academic libraries. Journal subscription prices are becoming increasingly prohibitive for developing nations, and the charges for single reprints online are becoming prohibitively expensive. Partial solutions to these problems include: searching for funds abroad from, for example, international agencies; improving the efficiency of institutional purchasing departments by educating people about the importance of scientific research for development; and relying on the support from international initiatives such as the Health InterNetwork Access to Research Initiative of the World Health Organization (see www.healthinternetwork.org), which is aimed at providing access to medical literature to developing countries, together with the help of colleagues and friends in developed nations that can provide electronic reprints.

IMPACT

This project has not yet reached the stage when human trials can be carried out. It is likely that the neutralizing properties of the fucoidan polysaccharide could be improved by more basic research studies using the preclinical mouse models, before beginning clinical trials (see below).

FUTURE PLANS

This project shows the potential therapeutic effect of fucoidan in counteracting local myonecrosis induced by PLA₂ myotoxins if rapidly administered *in situ*. The results are encouraging, but more studies need to be performed to improve the efficacy of this inhibitor or derivatives of it. For example, forms of fucoidan with lower molecular weights that would diffuse more quickly into tissues have not yet been explored. Likewise, forms of sulphated fucans, available from a large number of natural sources, also need to be evaluated. It will also be of importance to attract the interest of commercial companies that may be interested in developing fucoidan or related compounds as a therapeutic agent against tissue damage caused by snakebites.

In addition, collaborations currently exist between the Instituto Clodomiro Picado and clinical research groups in Latin America working on snakebite envenomations. In the future, it is hoped that such joint programmes will help the Institute to carry out clinical trials of the inhibitors in hospitals in areas with a high incidence of snakebites.

PUBLICATIONS

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