

Center for Inflammation Research

1. Name and full Postal Address of the Institution

Kalasalingam University
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Krishnankoil – 626 190
Tamil Nadu, INDIA

2. Project Title with a brief summary (not more than five lines)

Establishment of a Center for Inflammation Research

Scientists working in diversified fields of modern biology will be brought together to address various problems associated with inflammatory diseases; these scientists will bring together their expertise to understand the mechanism of pathogenesis and to develop novel therapeutic strategies for inflammatory diseases such as diabetic retinopathy, cancer and rheumatoid arthritis. Potential novel therapeutics from diversity of organisms will be explored.

3. Total cost of the project (phase wise break up)

Rs. 10 crores

4. Project Summary

a. Project Objectives

The above four interdisciplinary groups will work together with the following **Specific Aims**:

1. Understanding the molecular mechanism(s) underlying the pathogenesis of cervical cancer, diabetic retinopathy and rheumatoid arthritis in particular the therapeutic targets involved in signaling
2. Screening of anti-inflammatory compounds from diverse environments particularly the secondary metabolites of microbial origin; screening for enzymes which have antitumor activity from diverse environments
3. Bio-processing and optimization of the culture conditions for maximal production of the metabolite(s); Purification and characterization of the metabolite(s)
4. Elucidation of the mode of interaction of these ligands with their targets using bioinformatics tools.

b. Methodology to be adopted to achieve the objectives

Preliminary Work Plan

- 1. *Understanding the molecular mechanism(s) underlying the pathogenesis of breast cancer particularly the therapeutic targets involved in signaling***
 - a. Identification of potential therapeutic targets using gene profiling analysis*
 - b. Exploration of signaling molecules that can be used as therapeutic targets*
 - c. Cloning and expression of identified therapeutic targets*
 - d. Construction of secondary metabolite(s) production pathways in GRAS microbes or in cell lines*

- 2. *Screening of anti-cancer compounds from diverse environments, particularly secondary metabolites and enzymes of microbial origin***
 - a. Isolation of microorganisms from marine waters and invertebrate organisms*
 - b. Isolation from terrestrial environments particularly soil microbes from various regions of Western Ghats around Srilliputtur*
 - c. Screening of microbes for potential anti-inflammatory substances / anti-cancer enzymes such as asparaginase using a modified method of Woods, Sundar et al. (2007)*
 - d. Screening of microbes for anti-cancer enzymes such as asparaginase*
 - e. Further evaluation of anti-inflammatory substances for anti- cancer property using various cell lines*

- 3. *Bioprocessing and optimization of conditions for maximal production of the metabolite(s); Purification and characterization of the metabolite(s)***
 - a. Optimization of growth and production of anti-cancer substances using design of factorial experiments with tools such as Plackett-Burman design and Central Composite Rotary Design (Senthilkumar et al., 2005;2008).*
 - b. Modeling using genetic algorithms*
 - c. Estimation of production of anti-cancer metabolites under various conditions*
 - d. Purification of the enzyme / metabolites using chromatographic methods*
 - e. Scale-up studies*

4. Elucidation of the mode of interaction of these drugs with their targets using bioinformatics tools

- a. Structural elucidation of chosen metabolites
- b. Structural analysis and conformational analysis of chosen targets
- c. Molecular modeling and Docking using Discovery Studio
- d. Synthesis and modification of identified lead molecule(s)
- e. Evaluation of lead molecule-derived entities for anti-cancer activity

Objective 1: Understanding the molecular mechanism(s) underlying the pathogenesis of diabetic retinopathy, cervical cancer and arthritis particularly therapeutic targets involved in signaling

Gene Profiling to identify potential targets: Dr. Sangiliayndi's group and Dr. Sundar's group will adopt similar methodologies to profile gene expression in diabetic retinopathy and cervical cancer respectively.

Expression profile in DR: Initial studies will be performed *in vitro* conditions. Retinal endothelial cells grown at high glucose concentrations (30-35mM) would reveal any genes which are upregulated during diabetes. Expression of genes by analyzing the mRNA profile will be compared with that of a control which is grown at normal glucose concentration (5mM). mRNA isolated by standard procedures will be converted to cDNA and then cRNA. The total cRNA will be allowed to hybridize with the oligos in microarrays to find out the number of genes that are being upregulated at high glucose concentrations. The upregulated genes will be further confirmed by using real time PCR.

Cell Signaling

The retinal vasculature is profoundly affected in DR and the importance of vascular endothelial growth factor (VEGF) in DR is well documented. VEGF, which increases microvascular permeability, acts as an endothelial cell-selective mitogen and is thought to be a key factor in DR. Its level in the vitreous, correlates with the severity of the disease. However, the switch from quiescent to active vessels often involves not only an increase in inducers of neovascularization but also a decrease in the concentration of negative regulators of angiogenesis. One such natural inhibitor present in eye is PEDF secreted by

the retinal pigment epithelial cells in the media. However, the mechanisms for the protective effects of PEDF against DR are presently unclear. Therefore, we are interested in studying the effect of growth factors and angiogenic inhibitors, such as PEDF, on cell survival, proliferation, migration and tube formation in primary retinal endothelial cells. Further we would like to study the regulation of cell signaling pathways, particularly activation and inhibition of PI3/Akt, Fkhr, Src, MAP kinase in the presence and absence of AGEs, Epo, VEGF and PEDF under normal and hyperglycemic conditions using HREC cells.

Objective 2: Screening of novel anti-inflammatory compounds from diversified environments particularly secondary metabolites of microbial origin

1. Searching Marine Environment for Novel Therapeutic Compounds

Collection of Samples:

Snorkeling and skin diving methods will be used to collect marine invertebrates associated with coral reefs present in shallow water (less than 5 mts.). Scuba diving will be used to collect deep water associated organisms. Microorganisms associated with marine invertebrates and sediments will also be isolated. Samples will be collected at different seasons in order to collect diversified invertebrates and microorganisms producing compounds of potential therapeutic value.

Isolation and identification of micorganisms:

Microorganisms such as bacteria and cyanobacteria from marine environment will be isolated by serial dilution and plating. Standard marine agar medium such as Zobelle's marine agar will be used for the isolation of marine bacteria. Bacteria will be identified using colony morphology, pigment production and biochemical characteristics. Final identification will be done by molecular techniques such as 16S rRNA sequencing. The DNA isolated from each of these bacteria will be used for amplification of rRNA sequences using universal primers. Gene sequencing will be done by automated gene sequencer.

Isolation and Extraction of Conotoxins:

The present project also proposes to isolate conotoxins from species found in the Bay of Bengal, in and around the east coast. At present around 25 cone snails have been identified in the east coast and many more present are yet to be identified. Surprisingly the toxins of many of these snails have not been studied in detail except for a few. The snails will be brought to the laboratory alive. After identification of the species, venom will be extracted by the method of Cruz *et al.* (1987); the extracted venom can then be stored at -70° C.

Since these toxins contains a wide range of potentially important compounds, we propose to extract the venom from the cone snail, isolate different fractions, purify and characterize them to study their anti-inflammation and anti-analgesic actions *in vivo* and *in vitro* using the methods described below.

2. Screening of Metabolites

Screening of anti-inflammatory compounds:

Microorganisms isolated from diverse environments will be screened for the production of anti-inflammatory activity. Immunomodulatory effect will be assayed using inhibition of complement, classical and alternate complement pathway and by inhibition of lymphoproliferation.

Assay for anti-cancer activity:

Anti-cancer activity of selected compounds will be assayed using a set of various cell lines. The cell lines that will be used for screening includes T24, MCF7, ME-180, HT-29, MOLT-4, K562, SW-900, HT1080, G-361, PA-1, NB-EB, Rh-30 and SiHA. Use of these diverse cell lines will help in getting many compounds that may act on one or the other type of cancer.

These cell lines will be grown to confluency in DMEM with 10% FCS or any appropriate medium such as RPMI-1640. The cells will be split and 10^5 cells will be seeded on a fresh 6 wells plate. The cells will be treated with different compounds of interest in each

well. An untreated well will be used as a control. The ability of the compounds to induce apoptosis/ cell proliferation will be assessed using MTT assay and apoptotic tunnel assay.

Cyclooxygenase assay:

Cayman COX assay kit will be used for screening of compounds for COX-2 inhibition. This assay directly measures $\text{PGF}_{2\alpha}$ produced by SnCl_2 reduction of COX-derived PGH_2 . The prostanoid product is quantified via enzyme immunoassay (EIA) using a broadly specific antibody that binds to all major prostaglandin compounds.

Assay for enzymes with anti-cancer activity:

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Objective 3: Bioprocessing and optimization of conditions for maximal production of the metabolite(s); Purification and characterization of the metabolite(s)

The selected organisms which are found to overproduce interested secondary metabolites / enzymes will be transferred to the Process Development group for optimization of conditions for the production of metabolites or enzymes. Initial studies will be done in batch culture.

Optimization of Medium and Culture Conditions

Optimization of the cultivation medium and culture conditions for the process organism is of paramount importance in order to formulate a viable process. The cultivation medium should i) support reasonably good growth of the organism and ii) include ingredients that serve as precursors of the compound of interest. The latter requirement is greatly aided by some knowledge of the structure of the compound. If the compound is structurally similar to any other molecule whose biosynthetic pathway has been worked out, it is very probable that similar enzyme systems and precursors may be involved in

the synthesis of the compound in question (e.g. biosynthesis of β -lactam antibiotics). The optimization of medium and culture conditions for the compound of interest includes the following steps:

Basic studies on growth of organism and detection of the compound of interest

A medium containing a complex organic nitrogen source, carbon source and vitamins would be used for this. The choice of a rich medium and culture conditions would be guided by the nutritional and growth characteristics of the organism. The purpose of this is to determine the growth of the organism under well-defined, reproducible conditions. The culture supernatant would be appropriately tested at different time points of growth for the presence of the compound. This study would also reveal whether the compound is growth-linked (a primary metabolite) or non-growth-linked (a secondary metabolite).

Studies on variation of medium composition

The approaches adopted here would be dependent on the results of the previous studies.

- A) If the compound of interest is produced in detectable amounts and is also growth-linked, the strategy would be to enhance the growth rate of the organism by manipulating the nature and concentrations of the nitrogen and carbon sources.
- B) If the compound is not produced in detectable amounts, the basal medium may be supplemented with appropriate additives, keeping in mind the chemical nature of the compound of interest and the effect of these additives on the growth of the organism.
- C) If the compound is a secondary metabolite whose production is repressed by the energy source, a non-repressive energy source may be used in the medium (e.g. Maltose, Lactose or a fatty substance). Alternatively, the concentration of the repressive energy source may be closely controlled by incremental additions.

Studies on variation of culture conditions

Once results from the two previous studies have been obtained, environmental parameters such as pH, temperature and aeration may be varied and their effects on the production of the compound studied.

All the above studies will be carried out in stationary / shake flasks. If aeration has a positive effect on the production of the compound, baffled shake-flasks will be used. Also, the diameter of rotation of the shaker's platform may be increased to facilitate better aeration.

Formulation of medium for large-scale production

From an economic standpoint, use of microbiological media constituents mentioned in Section I is expensive. Therefore, attempts would be made to replace these constituents with cheaper and readily available materials that can serve as sources of organic nitrogen, carbon and vitamins. Ingredients such as corn-steep liquor, cane molasses and sulfite waste liquor have been used with success in various fermentation processes. Even if production of the compound drops to half that obtained using expensive media ingredients, the use of cheap media ingredients should be actively pursued. It is possible that this drop in productivity would be compensated for by improvements in process control in the fermentor (e.g. better mixing, aeration, and control of pH and temperature).

The cultures grown in optimized conditions will be used for the production of metabolites using a fermentor. In case of secondary metabolites, catabolite repression influences the choice of the reactors. As a preliminary design procedure, production experiments will be carried out in a seed reactor, which can then be shifted to continuous reactors.

Purification of compounds:

Selected metabolites

Objective 4: Elucidation of the mode of interaction of these drugs with their targets using bioinformatics tools

Discovery Studio will be used for modeling analysis. “Molecular Modeler” platform will be used for analyzing the structure of known targets from PDB coordinates obtained from PDB structure database. Initial work will be with known therapeutic targets such as kallikrein (1SPJ), a plasma protease (for DR), VEGF (for inflammatory diseases in general),

The structure of the newer chemical entity isolated from our studies will be used as a lead. Molecular docking studies will be performed using the lead molecule. Energy minimization techniques will be applied to obtain better derivatives from the lead molecule.

The derivatives obtained from *in silico* methods will be used to synthesize novel derivatives that could be used as potential drug molecules.

References:

Cruz, L.J., De Santos, V., Zafaralla, G.C., Ramilo, C.A., Zeikus, R., Gray, W.R., Oliver B.M. 1987. Invertebrate vasopressin/Oxytocin homologs: Characterization of peptides from *Conus geographus* and *Conus straitus* venoms. *J. Biol. Chem.* 262(33):15821-4.

c. Self assessment reflecting specific competence for undertaking the project

The project coordinator (KS) has many years of research experience in USA laboratories in the area of inflammatory diseases. He has developed assays that were used for medium throughput screening of anti-inflammatory compounds. Currently his laboratory is working on mapping CTL epitopes of cervical cancer; the group has already cloned the E6 and E7 and working on screening of marine microorganisms for the production of therapeutic enzymes like asparaginase that has anti-cancer property.

Projector Director and Group leader of Molecular Pathogenesis group Dr. G. Sangiliyandi has many years of experience in laboratories of international repute; he has established a diabetic retinopathy group at Kalasalingam University. His laboratory is funded by DBT, DST and ICMR. Basic protocols for studying cell-cell communication

are already standardized in his laboratory; which will form a foundation for the proposed study.

d. Stipulated period of completion

FIVE YEARS

5. Introduction

Inflammatory diseases, particularly cancer, are undeniably the largest contributor of morbidity and mortality in human population worldwide. Breast cancer is one of the devastating diseases affecting millions of women across the globe. Whereas, in developing countries like India, the cancer of the cervix is the leading cause of mortality. Despite enormous work done in this area, there is no permanent remedy for this disease.

Diabetes is a metabolic disease, affecting millions of people in India. Diabetic retinopathy (DR) a complication of diabetes in the retina of the eye leads to blindness if left untreated. At present there is no cure for the disease.

These two areas are the major focus of the proposed center. Faculty at the department of Biotechnology at Kalasalingam University (KLU) has diversified fields of research interests from molecular biology and immunology of inflammatory diseases to identification of novel therapeutic agents with anti-inflammatory activity. They are interacting with each other and complement their research expertise to form a network to work on inflammatory diseases particularly cervical cancer and diabetic retinopathy.

In the proposed center, the core area of focus would be on ***Mechanism of pathogenesis, diagnosis and treatment of inflammatory diseases*** particularly diabetic retinopathy and cervical cancer.

a. Present state-of-the art

CERVICAL CANCER

Cervical cancer is the second largest cause of female cancer death worldwide, only next to breast cancer, and causes annual deaths of about 288,000 accounting for 10% of all cancer related deaths in woman. According to the latest global estimate, 493,000 new cases of cervical cancer occur each year, of which, 409,400 (83%) occur in women of developing countries while only 84,400 cases occur in that of developed countries (Amin *et al.*, 2005).

Cervical cancer is preventable and its cure rate is 100% if it is detected at the pre-cancerous stage. Like many other cancers, cervical cancer has progressive developments and multiple phases that have been defined for both pre-cancer and cancer stages, from mild cervical intraepithelial neoplasia (CINI) to more severe degrees of neoplasia through microinvasive lesions and finally to invasive cancer (Burd, 2003; Anhang *et al.*, 2004). The current WHO recommendation is to evaluate visual inspection to identify early curable cervical cancer (Kitchener *et al.*, 1999). Papanicolaou (Pap) smear is the most commonly used cytological examination in cervical cancer screening. In total absence of routine Pap smear examination in developing countries, cervical cancer is detected too late which leads to death in almost all cases. Even with Pap screening programs, a significant number of women in developed countries die due to cervical cancer (Anhang *et al.*, 2004).

Causality of HPV to Cervical cancer

In November 1991, a workshop organized by the International Agency for research on Cancer and the World Health Organization officially concluded that, based on epidemiological and laboratory data, the association between HPV infection and cervical cancer was beyond reasonable doubt, and infection with HPV should be considered as cause for the development of cervical cancer (Bosch *et al.*, 1992). However, from the perspective of molecular biology, the awareness of HPV linking to cervical cancer had begun in the early 1980s (Burd 2003; Motoyama *et al.*, 2004). In multinational studies, the prevalence of HPV in cervical cancer was found to be over 99% (Bekkers *et al.*,

2004). Co-factors that further increase the risk of invasive cancer among HPV positive women include older age, long term use of contraceptive, high parity, and smoking (Anhang *et al.*, 2004).

Prevalence of HPV

HPV is one of the most common sexually transmitted infections (Bosch *et al.* 1995). The total prevalence of HPV infection in the world is about 630 million, with world-wide prevalence of cervical cancer of 2,274,000, while 1,300,000 in Asia. HPV 16 and HPV 18 are the most prevalent genotypes, accounting for 50 – 60% and 10-12% of the cervical cancer cases in most countries.

HPV Pathogenesis and Oncogenicity

HPV viral integration into the host genomic DNA plays a fundamental role in the progression from low-grade to high-grade cervical neoplasia (Ueda *et al.*, 2003). Integration of the HPV genome into host cells often results in inactivation of the E2 viral repressor protein, leading to overexpression of the viral E6 and E7 of HR-HPV genotypes, whose proteins have been shown to bind and inactivate normal cellular tumor suppressor gene products p53 and pRB, respectively. This interaction will affect DNA repair mechanism and normal cells cycle control, resulting in sustained cell growth, finally leads to malignant tumor development (Burd, 2003; Motoyama *et al.*, 2004; Hebner *et al.*, 2005).

National Status:

A very high risk of cervical cancer is observed in India. The reported age-standardized incidence rate (ASR) of cervical cancer during 1993-1997 ranges from 11-30 per 100,000 women in different regions of India (Parkin *et al.*, 1997). The incidence rates are particularly high in rural areas: ASR as high as 55/100,000 (Rajkumar *et al.*, 2000).

Though a slow and steady decline in cervical cancer incidence rates is observed in some urban populations, the rates are still high, particularly in rural areas, and the absolute number of cases is on the increase due to population growth (Sankaranarayanan *et al.*,

2001). India accounts for a quarter (126,000 new cases, 71,000 deaths around 2000) of the world burden of cervical cancer (471,000 new cases and 233,000 deaths) (Ferlay *et al.*, 2001). Control of cervical cancer by early detection and treatment is one of the priorities of the National Cancer Control Programme of India.

DIABETIC RETINOPATHY

The World Health Organization (WHO) estimates that in the year 2000, roughly 3% of the total world population had diabetes (this includes type 1 and type 2). In adults aged 20 years and over, around 5% had diabetes, and in those aged over 55 years, there were more than one in ten. Over 70% of the 171 million people with diabetes in the year 2000 lived in developing countries. The WHO estimates that by 2030 the number of people with diabetes will have increased to 366 million, with most new cases of diabetes occurring in people of working age in developing countries. By 2030, more than 80% of people with diabetes will live in what is currently a "developing country". One of the objectives of this centre is to raise awareness about the importance of diabetes and study the molecular mechanism of diabetes and diabetic retinopathy.

Vascular endothelial barrier dysfunction occurs in a large number of disease processes including diabetic retinopathy, stroke, pulmonary edema, myocardial infarction, inflammatory bowel disease, nephropathies, rheumatoid arthritis, and tumors. In these diseases, increased vascular permeability is associated with elevated levels of one or more growth factors or cytokines (Harhaj and Antonetti, 2004).

Vascular endothelial growth factor (VEGF) has received considerable attention as a tumor-secreted vascular permeability factor (Senger *et al.*, 1983). Indeed, VEGF has been implicated as an important permeability factor in multiple disease processes, including diabetic macular edema (Duh and Aiello, 1999). VEGF is 50,000 times more potent than histamine in inducing vasopermeability in the dermal vasculature (Senger *et al.*, 1990). Although VEGF is thought to play a major role in stimulating vascular permeability, this process undoubtedly involves multiple other factors as well, including inflammatory cytokines such as interleukin. The kinase activity of c-Src increases in an IL-1-dependent manner and the ectopic expression of c-Src B activation, suggesting the

involvement of c-Src in IL-1 signaling (Funakoshi-Tago *et al.*, 2003). Over expression of IL-1 β induces NV in the brain and causes retinal NV in transgenic mice.

Increasing attention is being focused on inhibitors of vascular permeability. Angiopoietin 1, for instance, has been found to have an impressive effect in blocking blood vessel leakage in animal models (Thurston *et al.*, 2000). Pigment epithelium-derived factor (PEDF) has recently emerged as a molecule that can regulate vascular permeability. PEDF is known to have strong anti-angiogenic effects *in vivo* (Bouck, 2002) and to regulate endothelial cell actions such as migration, proliferation, and survival *in vitro* (Dawson *et al.*, 1999; Stellmach *et al.*, 2001; Duh *et al.*, 2002). *In vivo* studies have demonstrated that PEDF blocks VEGF-induced vascular permeability in the retina (Liu *et al.*, 2004). In this study, we are interested to explore the anti-permeability properties of PEDF and to investigate the molecular mechanisms by which PEDF exerts its anti-permeability effects. Therefore, we propose to study the mechanism of vascular permeability in diabetic retinopathy. The results would help us to understand the mechanism of PEDF in diabetic retinopathy, thus providing a useful lead to identify potential targets for therapy in diabetic retinopathy.

Visual loss is often considered the most feared complications of human disease, other than death. In 2002, 124 million people world-wide had poor vision and 37 million were blind (WHO report). Visual loss primarily occurs from either proliferation of new retinal vessel (proliferative diabetic retinopathy) or increased permeability of retinal vessels (diabetic macular edema). Diabetic retinopathy is one of the most severe of the several ocular complications of diabetes. Advances in treatment over the past 40 years have greatly reduced the risk of blindness from this disease, but because diabetes is so common, retinopathy remains an important problem (Frank, 2004). The earliest clinical signs of diabetic retinopathy are microaneurysms, small out pouching from retinal capillaries and dot intra retinal hemorrhages. These signs are present in nearly all persons who have had type 1 diabetes for 20 years (Klein *et al.*, 1984) and in nearly 80 percent of those with type 2 disease. Patients with preproliferative retinopathy have an increase in the number and size of intra-retinal hemorrhages. This increase may be accompanied by

cotton-wool spots; both of these signs indicate regional failure of the retinal microvascular circulation.

Proliferative diabetic retinopathy involves the formation of new blood vessels that develop from the retinal circulation. New vessels can extend into the vitreous cavity of the eye and can hemorrhage into the vitreous, resulting in visual loss, and can cause tractional retinal detachments from the accompanying contractile fibrous tissue. Late in the course of the disease, new blood vessels may form within the stroma of the iris and may extend, with accompanying fibrosis, into the structures that drain the anterior chamber angle of the eye. This development blocks the outflow of aqueous humor, causing neovascular glaucoma, with a devastating elevation of the intraocular pressure.

Another vital change that can occur as diabetic retinopathy progresses is diabetic macular edema, which involves the breakdown of the blood-retinal barrier, with leakage of plasma from small blood vessels in the macula, the central portion of the retina that is responsible for the major part of visual function. This causes swelling of the central retina. Resumption of the fluid elements from the plasma leads to deposition of its lipid and lipoprotein components and the formation of hard exudates. In a large population based study, the incidence of macular edema over a period of 10 years was 20.1 percent in patients with type 1 diabetes, 25.4 percent in patients with type 2 diabetes who required insulin, and 13.9 percent in patients with type 2 diabetes who did not require insulin (Klein et al., 1995). The relatively selective loss of pericytes from the retinal capillaries is a characteristic lesion that occurs early in the histopathology of diabetic retinopathy. Normal pericytes are thought to have a contractile function that helps to regulate capillary blood flow. The loss of pericytes is followed by the loss of capillary endothelial cells. Apoptosis, or programmed cell death is thought to account for the disappearance of both types of cells. Since neurons in the retina have high metabolic requirements, the hypoxia that results from extensive retinal capillary cell death is probable stimulus for the increased expression of molecules that enhance the breakdown of the blood- retinal barrier which leads to vascular proliferation (Patz, 1982).

PEDF has been demonstrated to act as a neuroprotective agent in the eye (Tombran-Tink, 2003). The neuro-protective nature of PEDF is particularly important since neuronal degeneration is thought to play a role in DR (Antonetti *et al.*, 2002). PEDF has been demonstrated as a strong inhibitor of retinal neovascularization in mouse models. This gives PEDF an additional therapeutic benefit for diabetic retinopathy, in addition to its anti-vascular permeability effects. PEDF has been found to have significant direct inhibitory effects on cultured endothelial cells. PEDF was found to inhibit endothelial cell migration toward a large panel of angiogenic inducers, including VEGF and FGF-2, at a potency greater than that of angiostatin, endostatin and thrombospondin-1 (Dawson *et al.*, 1999 and Volpert *et al.*, 1999). Duh *et al.* (2002) showed that PEDF significantly reduces VEGF stimulation of retinal endothelial cell migration and proliferation.

In addition to its *in vitro* effects, PEDF has been found to be potent inhibitor of angiogenesis *in vivo* (Dawson *et al.*, 1999 and Volpert *et al.*, 1999). At least six labs independently demonstrated that PEDF delivery inhibits ocular angiogenesis in animal models. Systemic (Stellmac *et al.*, 2001 and Crawford *et al.*, 2001) or intraocular (Duh *et al.*, 2002) administration of PEDF protein inhibits retinal neovascularization in the mouse model of oxygen induced retinopathy. In addition, PEDF gene transfer, via adenovirus (Mori *et al.*, 2002; Gehlbach *et al.*, 2002; Demetriades *et al.*, 2003) or adeno-associated virus (Auricchio *et al.*, 2002; Behling *et al.*, 2002; Mori *et al.*, 2002; Gehlbach *et al.*, 2002) has been demonstrated to inhibit retinal and /or choroidal neovascularization. Since, PEDF had previously been demonstrated to have potent effects on endothelial cell actions *in vitro* and angiogenesis *in vivo*, a very logical question arised regarding the potential ability of PEDF to regulate endothelial cell barrier function and vascular permeability. Intravitreal injection of PEDF was found to significantly reduce retinal vascular permeability induced by VEGF in mice (Liu *et al.*, 2004).

Therapeutic Targets for Inflammatory Diseases

Cyclooxygenases:

Cyclooxygenases are a group of enzymes called as Prostaglandin H Synthases or COX contain both cyclooxygenase and peroxidase activities. COX catalyzes the first step in the

biosynthesis of prostaglandins (PGs), thromboxanes, and prostacyclins; the conversion of arachidonic acid to PGH₂. There are two distinct isoforms of COX viz., COX-1 and COX-2. COX-1 is constitutively expressed in a variety of cells and is involved in the normal cellular homeostasis. The expression of COX-2, the other isoform of COX is induced by a variety of mitogenic stimuli such as phorbol esters, lipopolysaccharides, and cytokines. COX-2 is responsible for the biosynthesis of PGs under acute inflammatory conditions (Xie *et al.*, 1991). This inducible COX-2 is believed to be the target enzyme for the anti-inflammatory activity of non-steroidal anti-inflammatory drugs.

Marine environment, a rich source of novel metabolites

Marine environment is believed to be an excellent source of many products that still remain unexplored. In the terrestrial environment, plants are believed to be the richest source of natural products. However in marine environment, this leading position is taken by invertebrates such as sponges, molluscs, bryozoans, tunicates etc. They not only produce a great number of currently known marine natural products, but also show the largest chemical diversity of natural products. The search for newer drugs from marine organisms resulted in the isolation of more or less 10,000 metabolites with diverse pharmacodynamic properties (Fusetani, 2000). Among marine invertebrates, porifera (sponges) remains the most prolific phylum concerning novel pharmacologically active compounds (Faulkner, 2000). Marine organisms such as sponges and the microorganisms associated with them are sources of many secondary metabolites that have diversified bioactivity. The diversity of secondary metabolites produced in sponges has been highlighted in several reviews (Sarma, Daum and Muller, 1993).

Lovell (1966) identified unique metabolites by x-ray crystallographic methods; they exhibited impressive *in vitro* antibiotic properties against gram positive bacteria with Minimum Inhibitory Concentration (MIC), ranging from 0.0063 to 0.2 g/ml. Bertrand and Vacelet (1971) reported that about 38% of the sponge body comprises microorganisms. A wide variety of secondary metabolites were isolated from sponges and these have been associated with antibacterial, antifungal, antiviral, antifouling, HIV protease inhibitory, HIV reverse transcriptase inhibitory, immuno suppression and

cytotoxic activities as well as to potential anticancer applications. Stierle *et al.* (1988) proved that the production of sponge metabolites is truly by microorganisms associated with the sponges. Sponges are also known to produce a potent cytotoxic compound, macrolactone swinholide (Carmly and Kashman, 1985; Kitagawa *et al.*, 1990). In 2004, Aneiros and Garatein reported that out of 11 genera of marine sponges screened for bioactive compounds, only 3 genera (Haliclona, petrosia and Discodemia) are known to produce anti-malarial, anti-cancer and anti-inflammatory compounds. *Bugula neritine*, a brown bryozoan animal with stringy tufts that look like algae, appears unremarkable and similar to a variety of moss-like sea creatures. Bryozoans are widely known by boat operators, who consider them ordinary fouling organisms and often scrape them off their vessels hulls. Fucans are sulphated polysaccharides extracted from brown seaweed, which display a wide scale of activities including inhibition of tumour cell invasion. Like several sulphated polysaccharides, they have been shown to be potent inhibitors of experimental metastasis.

Bryostatin-1 is a macrocyclic lactone and a potent activator of protein kinase C (PKC), and has antagonistic effects on tumor-promoting phorbol esters (Marshall *et al.*, 2002; Wender, 1986). Bryostatin-1 also has immunomodulatory functions, induces the differentiation of myeloid and lymphoid cell lines, platelet aggregation and promotes hematopoiesis (Thijssen *et al.*, 1999). Furthermore, bryostatin-1 inhibits the production of components of the matrix metalloproteinases family, down-regulates multidrug-resistance 1 (*MDR1*) gene expression, modulates *bcl-2* and *p53* gene expression and induces apoptosis (Thijssen, *et al.*, 1999). *Dolabella auricularia* is a mollusc from the Indian Ocean. Dolastatins are peptides isolated from *Dolabella auricularia*; the linear peptide dolastatin 10 and the desipeptide dolastatin 15 exhibit the most promising antiproliferative actions. The dolastatins inhibit cell proliferation and induce apoptosis in numerous malignant cell lines (Ojika *et al.*, 1995). These actions are mediated through interactions with tubulin, resulting in the alteration of microtubule function. Recent data indicate that the dolastatins also induce apoptosis in cancer cell lines. Halichondrins, spongistatin, curacin, laulimalide and discodermolide Halichondrin B, a macrocyclic polyether isolated from the sponge *Halichondria okadai*, *Elysia rubefescens*.

Discodermolide, a novel drug isolated from the marine sponge *Discodermia dissoluta*, works with paclitaxel to thwart tumor cell growth--with several times the efficacy that either drug alone exerts on proliferating cancer cells. *Psammaphysilla* sp a verongid sponge produces Psammaphin A is an antibiotic, anti-tumor and DNA methyltransferase inhibitor. It is a bromotyrosine-derived, symmetrical conjugate of cystamine, which was first isolated from the *Psammaphinaphysilla* sponge. Psammaphin A impedes angiogenesis as well as bacterial and tumor cell growth. Psammaphin A inhibits the activities of several key enzymes in prokaryotic and eukaryotic systems including those involved in epigenetic control of gene expression, DNA replication, angiogenesis, and microbial detoxification (Simmons *et al.*, 2005). Kahalalide F (KF) is a decapeptide isolated from the mollusk *Elysia rubefescens*. Kahalalide F (KF) is a novel antitumor drug of marine origin under clinical investigation. KF showed a potent cytotoxic activity against a panel of human prostate and breast cancer cell lines (Suarez, *et al.*, 2003).

Actinomycetes

Marine actinomycetes *Salinospora* sp (*Streptomyces* species) produces Salinosporamide A. Actinomycetes are a remarkably prolific source of structurally diverse secondary metabolites, including many that possess pharmaceutically relevant biological activities. *Streptomyces* species possess a single linear chromosome consisting of a conserved core flanked by two nonconserved arms (Asolkar, *et al.*, 2002). The arms of the chromosome contain largely acquired DNA and are the location of most contingency genes, including those that code for nonessential functions, such as secondary metabolite production. Thiocoraline is a novel bioactive depsipeptide isolated from *Micromonospora marine*, a marine microorganism that inhibits RNA synthesis (Romero *et al.*, 1997).

Symbionts

Tunicates grow on mangrove roots as symbionts (*Ectenaiscidia turbinata* associated with *Pseudomonas fluorescens*, *Endoectenaiscidia frumentensis*) (Erba *et al.*, 2001). They are producing Ectenaisdins (Ets) are tetrahydroisoquinolone alkaloids isolated from *Ectenaiscidia turbinata*, and they are mainly selected for clinical development because of their cytotoxic activity and relative abundance within the tunicate compared with others

Ets. ET-743 alters the interaction of DNA with transcription factors and other proteins (Jimeno *et al.*, 1996).

Bacteria

The potential low molecular weight anticancer agent *Cupredoxins* produced from *Pseudomonas aeruginosa*, *B. cepacia*, *Clostridium sp.*, *Salmonella sp.*, *Chromobacterium violaceum*, *Alkaligenes sp.* and *Paracoccus versutus* (Okvist *et al.*, 2002). The dynamical properties of the cupredoxins might be controlled for functional advantages that include the binding mechanism with the biological partners and the collective inner motions of the protein matrix required for the electron transfer. Dactinomycin, anthracyclines, mitomycin and bleomycin are anticancer agents derived from microbial sources.

Cyanobacteria

Cyanobacteria is one of the richest sources of known and novel bioactive compounds including toxins with wide pharmaceutical applications. Among the five divisions of microalgae, studies of biomedical natural products have been concentrated on only two divisions, i.e., Cyanophyta (blue-green algae) and Pyrrophyta (dinoflagellates) (Klisch and Hader, 2008). The role of bioactive molecules in the producer organism itself is poorly understood but, considering the wide spectrum of biological adaptations and tolerance to environmental stress revealed by cyanobacteria, some of these compounds can be produced in an attempt to confer advantages for their survival. Lyngbyatoxin-A and debromoaplysiatoxin are two highly inflammatory but structurally different metabolites isolated from toxic strains of *Lyngbya mausculata* and anatoxin-a from *Anabaena ciecinalis*. Some anti-HIV activity has been observed with the compounds extracted from *Lyngbya lagerhaimanii* and *Phormidium tenue*. More than 50% of the 100 isolates from marine sources are potentially exploitable bioactive substances. The substances tested for were either the ones that killed cancer cells by inducing apoptotic death, or those that affected cell signaling through activation of the members of protein kinase-C family of signaling enzymes. The anti-inflammatory and anti-proliferative properties of scytonemin, an extracellular sheath pigment originally isolated from the cyanobacterium, *Stigonema* spp. Goniodomin-A, an antifungal polyether macrolide from the dinoflagellate *Goniodoma pseudogoniaulax* has been shown to inhibit angiogenesis

by the inhibition of endothelial cell migration and basic fibroblast growth factor (bFGF)-induced tube formation and is active *in vivo*. An immunosuppressive linear peptide microcolin-A, which at nanomolar concentrations suppresses the two way murine mixed lymphocyte reaction, has been isolated from *Lyngbya majusculata*. A unique thiozoline-containing compound, curacin-A, has been purified from the organic extract of a Curacao collection of *L. majusculata*. This compound has been found to be an exceptionally potent antiproliferative agent as it inhibits the polymerization of tubulin, which shows some selectivity for colon, renal and breast cancer-derived cell lines. *P. hornemannii* is found to be a novel source of cytotoxic penta halogenated monoterpene, halomon, which exhibits one of the most extreme examples of differential cytotoxicity in the screening conducted by the National Cancer Institute (NCI), USA. Halomon has been selected for preclinical drug development since this compound shows toxicity to brain, renal and colon tumor cell-lines and preliminary *in vivo* evaluations have been encouraging. *Synechocystis* and *Synechococcus Anabaena*, *Microcystis*, *Oscillatoria (Planktothrix)*, *Nostoc* and *Anabaenopsis species* and terrestrial members of the *Hapalosiphon* are producing many novel therapeutics (Beltron and Nielan, 2000; Martins *et al.*, 2008)

Invertebrate Toxins as Therapeutic Compounds: Snails of marine environment are known to produce a variety of biologically active components. For example venom from a single cone snail can contain up to 200 biologically active components with different biological effects (Olivera *et al.*, 1990). These complex venoms are developed as a part of defense and feeding strategy. These peptides are directed against a wide variety of pharmacological targets, making them an invaluable source of ligands and used *in vivo* for proof-of-concept studies, with several having undergone preclinical or clinical development for the treatment of pain, diabetes, multiple sclerosis and cardiovascular diseases. Other toxins from dinoflagellates such as ciguatoxins, brevetoxins and diarrhoeic shellfish poisons exhibit a variety of pharmacological properties. Many of these toxins have proven to be invaluable research tools and have provided leads for potential new therapies. Most venom comprises a highly complex mixture of peptides, often with diverse and selective pharmacologic properties. Despite this diversity, venom peptides seem to have evolved from a relatively small number of structural frameworks

that are particularly well suited to addressing the crucial issues of potency and stability. It is this evolved biodiversity that makes venom peptides a unique source of leads and structural templates from which new therapeutic agents might be developed.

National Status:

Worldwide extensive researchers are undergoing on the venom of these snails, it has not yet attracted the attention of Indian researchers. Except few works on isolating the venom (28, 29), no attempt has really been made in testing the pharmacological and biochemical properties of Indian snails. Thus characterization of the Indian snail’s venom may unfold various bioactive substances, which meet the future needs of the biomedical discipline. On the contrary, unknowing the value, tons of these snails are being trawled during irresponsible fishing operation and dumped as trash in perished condition. This is seriously declining the resource of biomedically valuable mollusks.

References:

Xie, W., Chipman, J.G., Robertson, D.L., Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. Proc. Natl. Acad. Sci. USA. 88: 2692-2696 (1991).

b. Funding available from other sources

List of ongoing projects in the department of Biotechnology

<i>Title of the Project</i>	<i>Principal Investigator</i>	<i>Funding Agency</i>
<i>Molecular Mechanism of Vascular Permeability in Diabetic Retinopathy: PEDF is an anti-permeability agent</i>	<i>Dr. G. Sangiliyandi</i>	<i>DBT</i>
<i>Studies on Molecular Mechanism of Erythropoietin in Diabetic Retinopathy</i>	<i>Dr. G. Sangiliyandi</i>	<i>DST</i>
<i>Studies on Molecular Mechanism of Vascular Endothelial Growth Factor and Pigment Epithelium Derived factor in Diabetic Retinopathy</i>	<i>Dr. G. Sangiliyandi</i>	<i>ICMR</i>
<i>Scale up of polyhydroxyalkanoates production from indigenous isolates and its applications</i>	<i>Dr. G. Sangiliyandi</i>	<i>DBT</i>
<i>Bacteriophage – a novel vehicle for delivering HIV CTL epitopes</i>	<i>Dr. K. Sundar</i>	<i>LSRB, DRDO</i>
<i>New Deviation parameter methods to predict secondary structure of protein from amino acid sequences</i>	<i>Dr. S. Arul Mugilan</i>	<i>DST</i>

c. Level of infrastructure available

Basic equipment needed for the initiation of the work is available at the Department of Biotechnology, Kalasalingam University, Krishnankoil. The University will provide building infrastructure, furniture, electricity and water.

Biological safety cabinets	Micro centrifuge
CO ₂ incubator	Ice maker
ELISA reader	Platform Rocker
Fluorimeter	Fermenter
Amaxa Transfection Device	Fraction collector
Inverted microscope	Refrigerators
Deep freezers (-20°C and -80°C)	Sonicator
Autoclave	Magnetic stirrer
BOD Incubator	Electrophoresis Gel System
Cytospin	Semi-Dry transfer apparatus
Electronic balances	Cyclomixer /vortex mixer
High speed refrigerated centrifuge	Vacuum Pump
UV-VIS Spectrophotometer	Bacteriological Incubator

d. Nature of ongoing activities pertaining to project under submission in the institution

The laboratory of Dr. Sangiliyandi is working on the role of VGEF and PDEF in diabetic retinopathy. The laboratory has established protocols for the isolation of retinal endothelial cells and has completed preliminary studies in defining the role of these factors.

The laboratory of Dr. Sundar has initiated studies on cervical cancer and therapeutic enzymes of marine microbial origin. They have demonstrated the production of asparaginase over producing bacteria and standardized the conditions for the production

of enzyme. His group is involved in the cloning and expression of E6 and E7 proteins of Human Pailloma Virus which has implications in cervical cancer.

The laboratories of Dr. H. Nellaiah and Dr. S.R. Senthilkumar are working on optimization of conditions for the production of therapeutic enzymes of microbial origin.

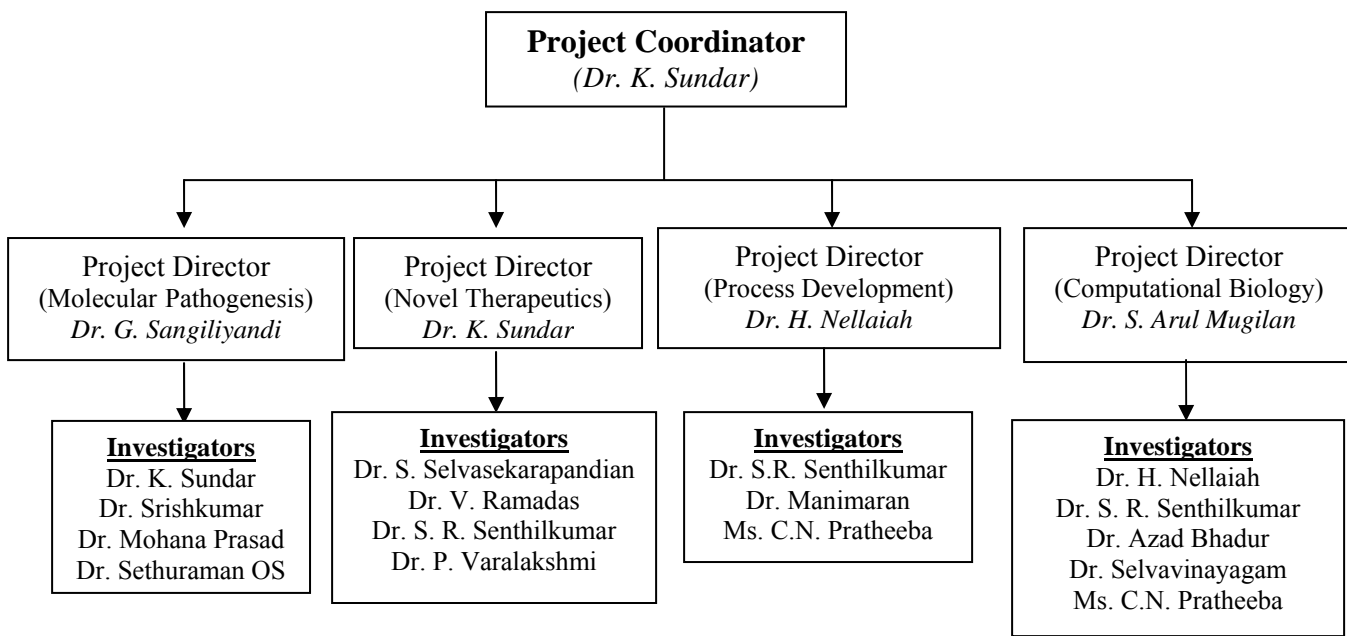
The laboratory of Dr. Arul Mugilan is working on the elucidation of secondary structures of proteins. He has expertise in Modeling softwares and bioinformatics programming. The laboratory of Dr. Sethuraman is working conotoxins which has therapeutic utility.

6. Budget (*Please see Annexure I and II for details*)

	I year	II Year	III year	IV Year	V Year	Total
a. Non-Recurring						
Equipments	11,12,85,000					11,12,85,000
b. Recurring						
Salaries						
JRF	21,60,000	21,60,000	25,20,000	25,20,000	25,20,000	1,18,80,000
Research Associate	9,60,000	9,60,000	9,60,000	9,60,000	9,60,000	38,40,000
Consumables	40,00,000	15,00,000	15,00,000	15,00,000	15,00,000	1,00,00,000
Softwares	60,00,000	--	--	--	--	60,00,000
Contingency	3,00,000	3,00,000	3,00,000	3,00,000	3,00,000	15,00,000
Travel	3,00,000	3,00,000	3,00,000	3,00,000	3,00,000	15,00,000
Total						14,60,05,000

7. Plan of Execution of project

a. Organizational set up



b. Principal Investigator with designation

Dr. K. Sundar, Professor of Biotechnology
 Dr. G. Sangiliyandi, Professor and Head
 Dr. H. Nellaiah, Professor of Biotechnology
 Dr. S. Arul Mugilan, Assistant Professor of Biotechnology

Please see Annexure III for bio-data of PIs

8. List of Research publications in concerned or allied areas

Please see Annexure IV

9. Existing facilities

Library:

Kalasalingam University has a central library with excellent collection of books and periodicals necessary for biologists.

10. Any linkage with other institutions

Project Director	Collaborator	Area of collaboration
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Dr. G. Sangiliyandi Professor & Head Department of Biotechnology	Dr. Soo Hyun Eom Professor Department of Life Sciences Gwangju Institute of Science and Technology Gwangju-500-712 Republic of Korea	Structural analysis of anti- inflammatory compounds
Dr. G. Sangiliyandi Professor & Head Department of Biotechnology	Dr. Cho, Jae-Youl Professor Division of Biological Engineering programme School of Biotechnology Kangwon National University Chuncheon, G(K)angwon 200-701 Republic of Korea.	Pathogenesis of Diabetic Retinopathy
Dr. K. Sundar Professor of Biotechnology	Dr. Mukundan Attur Director, Rheumatology Research Laboratory NYU Hospital for Joint Diseases School of Medicine, New York University New York, NY USA	Target identification; cell lines and assays for inflammation
Dr. K. Sundar Professor of Biotechnology	Dr. Richard Coico Senior Associate Dean for Research School of Medicine Temple University Philadelphia, PA USA	
Dr. K. Sundar Professor of Biotechnology	Dr. Muthusamy Kunnimalaiyan Senior Scientist and Director, Endocrine Surgery Research Lab School of Medicine University of Wisconsin Madison, WI USA	Cervical Cancer