1-1-2001

The effects of thymic hormones on the proliferation of human myelogenous leukemia cells

Melissa Pompilius
University of Nevada, Las Vegas

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

UMI®

THE EFFECTS OF THYMIC HORMONES ON THE PROLIFERATION OF HUMAN MYELOGENOUS LEUKEMIA CELLS

by

Melissa Pompilius

Bachelor of Science
Northern Arizona University
1998

A thesis submitted in partial fulfillment
of the requirements for the

Master of Science Degree
Department of Chemistry
College of Sciences

Graduate College
University of Nevada, Las Vegas
August 2001

UNLV

Thesis Approval
The Graduate College
University of Nevada, Las Vegas

July 9, 2001

The Thesis prepared by

Melissa Pompilius

Entitled

The Effects Of Thymic Hormones On The Proliferation Of Human Myelogenous Leukemia Cells

is approved in partial fulfillment of the requirements for the degree of

Master of Science

Examination Committee Chair

Dean of the Graduate College

Examination Committee Member

Examination Committee Member

Graduate College Faculty Representative

PR/1017-53/1-00

ABSTRACT

The Effects of Thymic Hormones on the Proliferation of Human Myelogenous Leukemia Cells

by

Melissa Pompilius

Dr. Bryan L. Spangelo, Examination Committee Chair
Associate Dean of the College of Sciences
Associate Professor of Chemistry
University of Nevada, Las Vegas

Thymosin fraction 5 (TF5), comprised of an array of small molecular weight peptides partially purified from the adult bovine thymus gland, contains multiple biologically active humoral factors. TF5 influences T cell differentiation, lymphokine production and restores immune deficiencies in a variety of animal models *in vitro* and *in vivo*. Because TF5 generally enhances immune reactivity, we examined the effects of TF5 on the proliferation of HL-60 human promyelocytic leukemia cells. Direct viable dye cell counting, the MTT reaction, and the clonogenic potential of HL-60 cells were used to determine cell proliferation. The potency and efficacy of TF5 for the inhibition HL-60 cell proliferation was similar in all three measures of proliferation. In contrast to HL-60 cells, TF5 only induced a modest suppression of human melanoma CRL7686 cell proliferation. Known apoptosis inhibitors did not reverse the effect of TF5 on HL-60 cell

proliferation indicating that TF5 acts through a non-apoptotic mechanism. The results of morphological analyses and the TUNEL assay demonstrate that TF5 does not induce apoptosis in HL-60 cells, suggesting that the mechanism of inhibition involves cytostasis. Further purification of TF5 by gel filtration chromatography produces a biologically active factor of approximately 20 kDa. Since the parent compound is composed of peptides less than 15 kDa, these results suggest the possible formation of oligomers. Electrophoresis of the active factor on polyacrylamide gels indicates that it contains at least four components in the 5 – 15 kDa molecular weight range.

These results demonstrate that an activity in TF5 exerts an anti-proliferative effect in human myeloid leukemia cells through a cytostatic mechanism; these studies suggest that a thymic hormone immune surveillance mechanism may limit the onset of certain types of leukemia.

TABLE OF CONTENTS

LIST OF FIGURES

ACKNOWLEDGEMENTS

I feel very thankful and blessed that I had the opportunity to attend graduate school and participate in this important research. I am deeply grateful to all of the people who helped to make this possible for me. I thank my parents, Jan and Gil Thomas, who have supported and encouraged me in every way that there is to help me reach this goal. I thank my daughter, Maia, for tolerating having a Mommy who is also a student. I am indebted and thankful to my advisor, Dr. Bryan Spangelo, for giving me the opportunity to work on this project, and for being a dedicated and supportive teacher, mentor, and friend. I thank Randy Calegari and Christian Samuelson for their hard work, commitment, friendship, and comic relief during the countless hours we worked together in the lab, and for all the times that they "had my back". I also appreciate the efforts of Jennifer Elbe, Jon Han, and Young Lee in the lab. I am indebted to our collaborators; Dr. Mahnaz Badamchian and Dr. Allen Goldstein who provided the TF5 and prepared the rpHPLC fractions, and Dr. David Jarvis who provided the cytological analyses and the HL-60 cell line. Dr. Goldstein also kindly permitted me to reprint Figure 1 in this manuscript. I thank Juanita Lytel for her guidance and advice over the past three years, and for helping me with all of the administrative obstacles so that I could concentrate on my studies and research. I thank Dr. Steinberg for the extensive help and advice he provided with my HPLC work and for letting me use his HPLC system. I also appreciate Dr. Gary and Dr. Carper providing the use of their equipment and reagents for

electrophoresis. I thank Dr. Lydia McKinstry, Dr. Ronald Gary, and Dr. Steen Madsen for serving on my thesis committee and providing detailed reviews and very helpful suggestions on the content and structure of my thesis. I thank my mother for helping me sort through and organize hundreds of references, for helping to troubleshoot computer problems, for "waiting on me hand and foot" as I sat chained to the aforementioned errant computer, and for helping me type this manuscript. I also appreciate the financial support of this research by the UNLV Graduate College through the G.R.E.A.T. Assistantship program.

CHAPTER 1

INTRODUCTION

Background

Thymus mediated immunity is vital for the preservation of immune function. Age and disease-related involution of the thymus is accompanied by parallel decreases in the levels of thymic hormones in the thymic epithelium and the periphery (Schulof et al., 1981, Berthiaume et al., 1999). As shown in Fig 1, thymic hormone decline is attended by a decrease in immune reactivity and a corresponding increase in the incidence of age related diseases (Goldstein et al., 1975). Furthermore, this decline is correlated with an increase in cancer incidence (Goldstein et al., 1975; Low et al., 1978). While the role of thymic hormones is poorly defined, growing evidence suggests that, together with cytokines, they act within the thymus and the periphery to influence T lymphocyte differentiation and therefore, immune function. Much of the effort to clarify the immunological role of thymic hormones has been performed using a protein extract of the bovine thymus termed thymosin fraction 5 (TF5). Several component polypeptides of TF5 have been isolated and purified, but not all of its observed biological effects can be ascribed to these. TF5 exhibits numerous biological activities that generally enhance immune reactivity (Reviewed in Low et al., 1979; Spangelo et al., 1997). Further, previously reported results from our laboratory demonstrated that TF5 is an effective

inhibitor of proliferation in rat neuroendocrine tumor cells (Spangelo et al., 1998, 2000). Other studies have shown that TF5 inhibits the growth of some cancer cells *in vitro*, including certain murine leukemia cells (Khaw et al., 1973; Schultz et al., 1976; Petro et al., 1982) and human acute lymphoblastic T cell leukemia cells (Ho et al., 1983, 1983a). TF5 has also been shown to normalize the *in vitro* mixed lymphocyte response (MLR) responses of T cells from leukemia patients.

Because TF5 generally enhances immunity and can moderate tumor activity, and because cancer is generally accompanied by impaired cellular immunity, we tested whether TF5 would inhibit the growth of human HL-60 acute myelogenous leukemia cells *in vitro*.

To clarify the relationships between HL-60 cells, cellular immunity, and thymic hormones in the context of this study, pertinent aspects of several topics are reviewed briefly: the immune system, the biology and chemistry of thymic hormones, leukemia and the origin of the HL-60 cell line, and thymic peptides as immunotherapeutic adjuvants.

Immune System

The white blood cells of the human immune system arise in the bone marrow and circulate in the blood and lymph. In circulation, they function cooperatively to defend the body against infection and cancerous body cells. Hemopoietic stem cells in the bone marrow originate three principal classes of white blood cells: phagocytes, B-cells, and T-cells. Phagocytes leave the bone marrow and become participants in immediate defense and activation of the immune response. These cells, including macrophages and their

monocyte precursors, engulf pathogens and enzymatically degrade them. Antigen presenting cells, APC's, are macrophages that display protein fragments of engulfed cells to facilitate immune system recognition of antigens. Some phagocytes become lymph-tissue-associated defenders in the liver, spleen, lymph nodes or other tissue. Here, they trap cellular debris and foreign particles, removing them from circulation (Raven and Johnson, 1992). Others become mobile defenders circulating in the blood and lymph, where they phagocytose circulating pathogens that have not yet infected cells. Cytokines such as interleukin-3 (IL-3), interferon-γ (IFN-γ), tumor necrosis factor α (TNFα), and IL-4 secreted by infected cells and T- cells activate macrophages at local sites of infection. Upon activation, macrophages in turn secrete cytokines such as IL-1, IL-6, IFN- γ and TNFα, which signal the onset of the immune response (Aspinall, 1997). IFN-γ promotes the development of monocytes into additional macrophages for defense. Secreted IL-1 activates T-cells that have been stimulated by infected cells and prepares them to proliferate (Raven and Johnson, 1992). T-cells, upon activation by macrophages, initiate two parallel immune responses: the humoral immune response and the cell-mediated immune response.

The humoral response is the long-range antibody defense orchestrated by the B-cells. B-cells in the bone marrow develop cell-specific receptors on their surfaces that allow them to recognize particular foreign antigens. They are then released into the bloodstream and lymph where they differentiate into plasma cells after encountering pathogens. This is accompanied by protein antigen uptake and presentation on the B-cell surface in combination with major histocompatibility complex II (MHC II) molecules. This complex can then be recognized by antigen specific helper T cell receptors (Raven

and Johnson, 1992; Aspinall, 1997). Activated helper T cells (T_H) and macrophages (APC's) also secrete cytokines that induce B-cells to produce large clones of plasma cells (Raven and Johnson, 1992). The plasma cells secrete specific antibody molecules that bind antigens and flag the antigen-bearing cells or viruses for destruction.

The cell-mediated immune response involves the destruction of these flagged cells and abnormal (*i.e.* cancerous) host cells. To achieve this, T_H cells secrete a variety of cytokines such as IL-2, Macrophage Migration Inhibitory Factor (MMIF), IL-1, IL-4, and IL-5. IL-2 induces the proliferation of both cytotoxic T cells (T_C) and additional T_H cells. Activated T_C cells express specific receptors that recognize the antigen- MHC molecule complex on antigen presenting (*i.e.* dendritic) cells and bind them, inducing death by lysis. Depending on the cytokine environment, T_C cells can alternatively induce apoptosis in antigen-bearing cells (Aspinall, 1997). Macrophage Migration Inhibitory Factor retains macrophages at infection sites (Raven and Johnson, 1992). IL-1, IL-4 and IL-5 participate in B-cell activation (Aspinall, 1997). T_H cells also secrete cytokines that trigger the development and differentiation of T-cells.

This T cell maturation takes place in the thymus, a primary lymphatic tissue located in the upper thorax just above the heart. The function and physiological role of the thymus were not known until the 1960's when studies established that thymectomy or impaired thymic development in newborn animals (*i.e.* dogs, guinea pigs, rodents) and humans results in decreased lymphocyte numbers in the tissue and blood, as well as failure of immunological competence. These studies led to the recognition that primary transplant, tumor, microbial, and fungal immunities are under thymic control in mammals (Low and Goldstein, 1978).

The human thymic core is formed by a fusion of two different types of embryonic tissue, the 3rd pharyngeal pouch endothelium and the 3rd branchial cleft ectoderm. Studies in embryonic mice indicate that the ectodermal layers generate the epithelial cells in the thymic cortex, while the endodermal layers produce the epithelia of the medulla (Roitt, Brostoff and Male, 1993). In athymic (nude) mouse embryos, this joining of ectoderm and endoderm is absent, and the thymus never becomes functional (Kendall, 1991). Early in the development process, the thymic foundation formed by these two layers is divided into two glands by membranes (septa) from the surrounding mesenchyme. This division allows the entrance of nerves, blood vessels, and other cells into the cortex and medulla. Monocytes enter and differentiate into macrophages that populate the cortex and corticomedullary junction, or into interdigitating cells (IDCs) that reside in the medulla (Kendall, 1991). All of these cells, along with the thymic epithelial cells, display peptides associated with class I and class II MHC molecules, proteins central to T cell recognition of antigens. The thymus is largest relative to body weight at birth, and may gradually be reduced in weight with age, as the bulk of the thymopoietic tissue is replaced with fat cells in the process of "thymic involution" (Kendall, 1991). The mature thymus provides the microenvironment that controls the development of immature T-cells, also called thymocytes, into immunocompetent T-cells.

Prothymocytes from the bone marrow enter the thymus devoid of the molecules characteristic of mature T-cells: the CD3- T-cell receptor complex, and the CD4 or CD8 co-receptors. These double negative ($CD4^-/CD8^-$) cells populate the subcapsular region, where they proliferate rapidly and undergo gene rearrangement to express the cell surface receptor and both the CD4 and the CD8 co-receptors. The resulting double- positive cells

populate the cortex where they undergo positive and negative selection. Approximately 90% of the double positive cells express receptors that cannot recognize self-MHC molecules. These fail positive selection and undergo cell death by apoptosis. The remaining positively selected, self-restricted thymocytes occupy the cortex and corticomedullary regions, and have increased T-cell receptor expression accompanied by loss of expression of either CD4 or CD8. Single-positive cells then undergo negative selection, mainly in the corticomedullary junction, to eliminate strongly self- reactive thymocytes. Mature T- cells that are self-tolerant and self-restricted leave the thymus via the corticomedullary venules or lymphatics. The selection process requires extensive interactions between thymic stromal cells (*i.e.* macrophages, IDCs, and at least six subtypes of thymic epithelial cells) and thymocytes involving cytokines (IL's 1, 2, 6, 7, 8, IFN- γ), and thymic hormones (Bodey, et al., 2000). Two thymic factors that have been isolated from a partially purified extract of the bovine thymus (thymosin) have been localized in human thymus glands. Cells lining the cortical surface contain both $T\alpha_1$ and $T\beta_3$, whereas only $T\alpha_1$ can be detected in thymic epithelial cells (TEC) of the medulla. Further, several peptide components of thymosin can be detected in TECs and macrophages in the human thymus cortex and corticomedullary junction (Hirokawa, McClure, and Goldstein, 1982; Schuurman et al., 1985). The location of these peptides in the thymus, together with experimental data discussed later in this text (Hu, Low and Goldstein, 1981) indicates that they participate in specific stages of T cell development. The location of thymosin peptides within the cells of the thymus is illustrated in Fig 2. T-cell maturation peaks during the first year of life, and declines during thymic involution triggered by aging, acute injury, pregnancy, chemotherapy and other forms of stress

(Berthiaume, et al., 1999; Kendall, 1991). Concurrent with this decline in thymic function is a reduction in the secretion of thymic hormones, which have been shown to promote T-cell maturation and to modulate peripheral T-cell responses (Goldstein et al., 1975; Berthiaume et al., 1999).

Thymosin Fraction 5: Purification and Characterization

The thymic hormones represent a large heterogeneous group of polypeptides that includes the thymosins, thymulin, thymopoietin and a number of less well-characterized peptides. The role of these peptides is poorly defined but several lines of evidence suggest that together with cytokines they exert significant actions within the thymus and in the periphery that influence T lymphocyte differentiation and hence, immune function (Spangelo and Gorospe, 1997). The search for putative thymic hormones began as early as 1940, when thymic extracts were shown to stimulate the function and development of lymphoid tissue and to moderate the effects of thymectomy in a variety of animal models (Low and Goldstein, 1978). Since that time, the biological activities of several thymic extracts and their derivatives have been tested in both normal and thymectomized animals and in human clinical trials (Oates, Sztein and Goldstein 1989; Spangelo, 1995). The results of early attempts to isolate and purify thymic factors that could replace thymus-dependent immunity in normal and thymectomized animals were reported by Goldstein and colleagues in the mid- 1960s (Klein, Goldstein and White, 1965, 1966).

Thymosin, a stable, biologically active bovine thymus fraction, was identified in 1966, and purified to homogeneity in 1972 (Goldstein, Slater and White, 1966; Goldstein

et al., 1972). In 1975, Goldstein and colleagues developed a modified purification procedure that allowed the preparation of large amounts of thymosin extract, thymosin fraction five (TF5) (Hooper et al., 1975). The ability to produce significant quantities of the extract facilitated more extensive studies of its biological and chemical properties.

TF5 contains at least twenty heat stable polypeptides with molecular weights in the range of 1200-14000. TF5 can be separated on IEF gels into several components. A nomenclature system based on the resulting isoelectric patterns has divided TF5 into three bands. Peptides with isoelectric points (pI) of less than 5.0 are known as α-peptides (acidic). Those with pIs of 5.0 to 7.0 are the β-peptides, and those greater than 7.0 are γ-peptides. Subscript numbers (*i.e.* $T\beta_3$, $T\beta_4$) identify the peptides from each band in the order in which they are isolated (Low et al., 1979). Three peptide components, $T\alpha_1$, $T\beta_4$, and MB-35 have been purified and sequenced (Low, et al., 1979; Low and Goldstein, 1984). $T\alpha_1$ is a highly acidic (pI 4.2), 3108 MW peptide consisting of 28 amino acids residues. $T\beta_4$ has a MW of 4982, a pI of 5.1 and consists of 43 amino acid residues. Both of these peptides have been proposed to be thymic hormones. Peptide MB-35 is a basic molecule of 35 amino acid residues with a MW of 3756 (Badamchian et al., 1991).

Thymosin is 99% protein with a small amount of carbohydrate and a minute amount of nucleotide. Analysis of acid hydrolysates indicates an amino acid composition high (approximately 50%) in acidic residues, with very few (~ 3%) aromatic residues, and no tryptophan. Initial studies demonstrated its biological activity in murine systems designed to measure lymphocyte function. Thymosin partially restores the responses of congenitally athymic mouse lymphocytes to mitogens (Con A, PHA), increases survival times and immune responses to skin allografts (graft versus host reaction, GvH) in

neonatally thymectomized mice, induces T cell production in adult thymectomized mice and enhances the responses of lymphocytes from normal healthy mice to T and B cell mitogens. Thus, TF5 acts in place of the thymus in thymectomized or athymic animals and boosts immune parameters of healthy animals *in vitro*. Further, thymosin circulates in the blood of a variety of mammals. Serum thymosin activity in humans measured by a radioimmunoassay (RIA) shows lower levels with age, and with the presence of immunodeficiency disease, Hodgkin's disease, or leukemia (CLL) (Hooper et al., 1975).

TF5: Immune System Stimulation

Subsequent studies have shown that peptide components of TF5 participate in diverse immune functions. In animal models *in vitro* and *in vivo*, TF5 influences thymocyte development and T cell differentiation, induces lymphokine and cytokine production, stimulates and restores cellular and humoral immune function, increases disease resistance and moderates tumor activity. It has similar effects in human models *in vitro*. TF5 is not universally successful; its effects appear to be dependent on species, strain, age, health, and basal immune reactivity of test animals as well as on type of disease or tumor investigated and TF5 dose and treatment regimen. Clinically, TF5 increases resistance to certain diseases, restores immune parameters during disease states and moderates the activity of some tumors. Its ability to boost immunity and thwart disease and cancer activity make it therapeutically attractive for the treatment of certain diseases. Its efficacy *in vitro* may be an indicator of whether it will have therapeutic success for a particular disease.

Thymosin is extensively involved in thymocyte development and differentiation *in vitro*. It induces the differentiation of mouse thymocytes cultured with allogeneic spleen cells in the presence of antigen (Cohen, Hooper and Goldstein, 1975) and also induces T cell surface markers (TdT) in murine bone marrow stem cells *in vitro* (Pazmino, Ihle and Goldstein, 1978). Receptors for thymosin are present on rat thymocytes, particularly on cells with medium-sized nuclei rich in condensed chromatin and on dividing cells (Brelinska and Warchol, 1982). Incubation of rat cortical thymocytes with TF5 significantly increases the percentage of rosette forming cells (RFC) in this non-RFC rich population. In contrast, the RF percentage of medullary thymocytes, which generally do not form rosettes, is not affected by TF5. Spleen fraction 5 has no effect on either population (Kinoshita, et al., 1986). Additionally, thymosin and its component peptides $T\beta_3$ and $T\beta_4$ impact various thymocyte maturation sequences *in vivo*, such as TdT expression in murine bone marrow cells, and both the early and terminal stages of differentiation (Pazmino et al., 1978; Hu et al., 1981). It also induces maturation- stimulated expression of mature T cell markers in normal human thymocytes (Ho et al., 1983a).

In addition to stimulating differentiation of prothymocytes and thymocytes, pre-incubation with TF5 stimulates mature T cell activity and lymphokine production by murine cells *in vitro* (Zatz and Goldstein, 1985). Peripheral blood lymphocytes (PBL) from thymectomized guinea pigs sensitized with purified protein derivative (PPD) lose the capacity to produce MMIF. Normal intact PPD sensitized guinea pig PBLs do not experience this suppression. TF5 restores MMIF production in thymectomized animals but has no effect on normal intact animals (Thurman et al., 1984). Interleukin 6 (IL-6) is

a pyrogenic cytokine that stimulates pituitary hormone release and participates in T cell and B cell differentiation and the induction of IL-2 (T cell growth factor) and IL-2 receptor (IL-2R) expression. Thymosin in the presence of mitogen causes a synergistic increase in IL-6 production by rat spleen cells *in vitro*, an activity that is not due to the $T\alpha_1$ or $T\beta_4$ peptide components of TF5 (Attia et al., 1993). Previously published data from our laboratory suggests that a novel peptide component of TF5 enhances rat glial cell production of IL-6 *in vitro*, and may participate in IL-6-mediated inflammatory responses (Tijerina et al., 1997).

Similar effects have been observed in human cell cultures. In the presence of mitogen, TF5 enhances IL-2 production by normal human peripheral blood lymphocytes (PBL) (Zatz et al., 1984, 1985a; Sztein et al., 1986), an effect that is not due to Tα1 or Tβ4 (Zatz et al., 1984, 1985a). In comparison with other well-characterized thymic extracts, only one, a closely related porcine preparation exhibited similar activity to TF5 (Zatz et al., 1984, 1985a). The preparation also enhances IL-2R expression. This appears to be a direct effect of TF5, since nullification of IL-2 production does not affect the outcome (Sztein et al., 1986). TF5 and Tα1 enhance the mitogen-induced high affinity IL-2R expression by human lymphocytes (PBL) *in vitro*. This effect is coupled with increased levels of IL-2 production (Leichtling et al., 1990). Thymosin modulation of the IL-2/IL-2R system *in vitro* involves enhanced IL-1 production by macrophages (Sztein et al., 1989; Hu et al., 1989). In combination with aspirin (acetylsalicylic acid), the induction of IL-2 synthesis by human PBL is additive, and appears to be mediated by monocytes (Zatz et al., 1985b). Studies have shown that aspirin and TF5 also induce IFN-γ production *in vitro* and *in vivo* in healthy donors. Together, their effects are additive,

and both require the presence of macrophages to effect the stimulation (Hsia et al., 1989; Diezel et al., 1984).

TF5, but not kidney fraction 5, stimulates the spontaneous natural killer cell (NK) activity of human lymphocytes *in vitro*. The stimulation of NK activity is additive in combination with recombinant IFN-γ (Serrate et al., 1987). In addition to increased IL-2 synthesis, B cell growth factor (BCGF-12kD) production by human PBMC is enhanced by TF5, reinforcing the evidence that thymus peptides participate in the regulation of humoral (*i.e.* B cell) immune responses (Kouttab et al., 1988). Furthermore, TF5 administration *in vivo* restores the thymus-dependent enhancement of humoral immunity by estrogen in adult thymectomized rats, indicating that a hormone product of the thymus contained in TF5 enhances a specific humoral immune response (Erbach et al., 1991).

TF5: Restoration of Immune Deficiencies

TF5 also stimulates and restores cellular and humoral immune deficiencies resulting from age, radiation injury, protein deficiency, certain diseases and cancer *in vitro* and *in vivo*.

Mice can be chemically thymectomized by hydrocortisone treatment, resulting in loss of thymocyte and lymphocyte production. Treatment *in vivo* with mixed interleukins (*i.e.* IL-1, IL-2) prepared from mitogen-stimulated human buffy coat lymphocytes (BC-IL; buffy coat interleukins) increases the spleen and thymus weights and cellularity, and restores lymphocyte subsets and function in chemically thymectomized aged mice. TF5 substantially increases the promotion of T lymphocyte development by mixed BC-IL in

hydrocortisone treated aged mice *in vivo*, indicating that the two act together to promote T lymphocyte development (Hadden et al., 1992).

New Zealand Black (NZB) mice show an age-related loss of T cell functions and spontaneously develop an autoimmune and lymphoproliferative disease after 16 weeks of age. Abnormal thymocyte proliferation in these animals is prevented by daily intravenous injection of TF5 (Dauphinee et al., 1974). NZB mice also display an accelerated loss of antigen-induced depression of spleen DNA synthesis that can be restored *in vivo* by injection of young NZB thymocytes. TF5 treatment also restores this loss for 1-2 weeks *in vivo*. The restoration is repeatable with further TF5 treatment (Dauphinee and Talal, 1975).

In an age comparison study of human cells, T cells from normal, healthy older (65-103 years) subjects had lowered immune reactivity (MLR) *in vitro* as compared to young adults (22-41), although both groups had similar peripheral blood T cell counts. This decreased reactivity in the aged group was related to an inherent defect in the ability of the T cells to respond to mitogen. TF5 did not enhance the rosette-forming capacity of T cells (TCR) in either group. However, incubation with TF5 increased the MLR responses of both groups similarly. These results suggest that the decline in lymphocyte function with age is not due to a decrease in the population of thymosin-responsive cells (Cowan et al., 1981).

Recovery from radiation injury in mice requires thymus-dependent immunity. When adult thymectomized, sub-lethally irradiated mice were injected with thymosin, percent survival and survival time were both increased significantly over spleen extract and saline treated animals, suggesting that thymosin accelerates thymocyte development

in absence of the thymus (Hardy et al., 1971). The effect of thymosin on the recovery of mice from radiation is dependent on the strain and age of mice treated. In three diverse strains (C57B1/KsJ, C57B1/6, C3H/HeJ, DBA/1) that were radiation injured, all young (<13 wk) animals showed increased thymocyte responses to IL-1. In the DBA/1 strain, older animals (>5mo) did not respond to TF5, while age matched C3H/HeJ mice did. These results indicate that thymic hormones accelerate the immunological recovery of irradiated murine thymocytes in an age and strain dependent manner (Neta et al., 1985, 1985a).

Protein deficiency impairs both humoral and cellular immunity. A study of the immuno-restorative potential of TF5 demonstrated that it exerts differential effects on T cell functions in variably protein- deficient mice. Well-nourished (normal) mice injected with phosphate buffered saline (PBS) had significantly increased T cell and B cell mitogenic responses. PBS treated mice fed low and zero protein diets had smaller increases in induced mitogenesis. Introduction of TF5 treatment in the normal diet group gave small increases in mitogenic responsiveness. In the low protein group, TF5 stimulated mitogenic responsiveness to the levels of the control group. In the zero-protein group, TF5 did not have a significant effect on mitogenic response (Watson and Lim, 1986). In humans, lymphocytes from severely malnourished children had higher RFC percentages when incubated in the presence of TF5, indicating the presence of immature thymocytes in circulation in these patients and suggesting a potential therapeutic application for TF5 in malnutrition (Keusch et al., 1987).

Uraemic animals have increased thymic involution, decreased lymphocyte counts, and impaired mitogenic responses. When TF5 was administered to severely uraemic rats,

both thymus weight and lymphocyte populations were restored, suggesting that TF5 can restore suppressed thymic hormone secretion *in vivo*. (Ikemoto et al., 1992). Pretreatment with TF5 also improves the reduced response of thymocytes from severely uraemic rats to Con A *in vitro* (Ikemoto et al., 1990). In contrast, TF5 enhances the *in vitro* mitogenic response of immature lymphocytes from diabetic rats but decreases the mature T cell responsiveness (Okimoto et al., 1988).

TF5 restores several depressed immune parameters resulting from disease conditions and autoimmune disorders in humans, including lymphokine production, mitogenic responsiveness, and T cell differentiation. For example, IL- 2 levels in HIV positive pre- AIDS patients were reduced to approximately 20 percent of those in normal subjects. IL-2 production increased to 35% of normal levels when mitogen- stimulated peripheral blood lymphocytes from pre- AIDS patients were incubated with TF5. Tα1 had no effect (Skotnicki, 1988). In a similar population of patients, TF5 also increased MLR responses and stimulated mitogen-induced (PHA, PWM) IL-2 synthesis in peripheral blood mononuclear cells (PBMC) from patients with impaired cell mediated immunity. These effects are additive when combined with LiCl (Sztein et al., 1987).

In a study of patients with one of several lymphoproliferative disorders, T cells from patients with systemic lupus erythematosus (SLE) gave increased immune responses (*i.e.* mitogenic stimulation, T cell colony formation, and erythrocyte rosette formation) in the presence of TF5 as compared to those from healthy donors. Mitogenic responses were increased by TF5 in patients with lymphoproliferative disorders (LPD) (Hazama et al., 1978). Additionally, incubation with thymosin enhanced immune

reactivity and induced CD2 receptor expression of peripheral T cells from severely immunodepressed allogeneic marrow transplant patients (Elfenbein et al., 1980).

TF5 restores the suppressor activity of lymphocytes from chronic hepatitis patients *in vitro*. Patients with active chronic hepatitis (CAH) had significantly reduced suppressor cell activity compared to normal subjects. When lymphocytes from both groups were stimulated with mitogen in the presence of TF5, the cells from the CAH group had heightened suppressor activity whereas the normal subjects showed no effect (Mutchnik et al., 1983). Similarly, pre-incubation with TF5 normalized suppressor cell activity in peripheral blood lymphocytes from patients with rheumatoid arthritis (Zatz et al., 1984a).

TF5 also reinstates the loss of immune reactivity due to cancers. Oral administration of N-butyl-N- (4-hydroxybutyl) nitrosamine (BBN) induces urinary bladder cancer in rats. This results in a gradual decline in lymphocyte rosette forming capacity and a progressive reduction in MLR and mitogenic responsiveness of the rat thymocytes *in vitro* that peaks during the hypertrophic stage preceding carcinoma incidence. Pretreatment of lymphocytes with TF5 restored RFC to near control levels (Kimura, et al., 1986), and improved mitogenic activity (Wada et al., 1987). Responsiveness was also restored in thymocytes pretreated *in vitro* with TF5. The restorative effects were greater in the hyperplastic stage and early in tumor development (Asakawa et al., 1990).

In a study that tested the *in vitro* effects of thymosin as an immunotherapeutic adjunct in humans, cells were taken from patients with disseminated cancers and known, limited survival times that had received thymosin injections. Patients in this study

received no other forms of immunotherapy, and thymosin administration was not given on the same days as chemotherapy. *In vitro* tests measured the blastogenic and MLR responses of lymphocytes with and without thymosin. These data were compared to responses obtained from lymphocytes taken from normal subjects. In both cases, increased T cell response to thymosin was associated with depressed basal levels of T cell activity while decreased responses were obtained from subjects with elevated basal activity. Thus, thymosin tended to normalize lymphocyte responses *in vitro* without acting as a mitogen or antigen. Clinically, anecdotal observations suggested patients with leukemia (ALL, AML, CLL), malignant melanoma and multiple myeloma appeared to benefit from thymosin therapy, whereas accelerated disease appeared during thymosin treatment in a male patient with choriocarcinoma. No systematic clinical trial was conducted, however. Skin test reactivity to antigens was increased in approximately forty percent of patients, and gradually declined over a 1-2 month period post treatment. This study indicates that thymosin may have potential as an antitumor agent in leukemia treatment, that its immunotherapeutic benefit is related to depressed basal lymphocyte function, and that *in vitro* responses may be useful in evaluating candidates for thymosin therapy (Schafer et al., 1976).

TF5: Direct Effects on Disease And Cancer

In addition to restoring disease and cancer- related immune deficiencies, TF5 also directly affects disease and tumor progression. Shortly after Goldstein and colleagues reported their initial findings that thymosin enhanced T cell functions *in vitro* (mitogenic response, GvH, differentiation), it was tested in the treatment of amyloid disease (e.g.

Alzheimers) in mice *in vivo*. This study was logical, since the pathogenesis of amyloid disease has been related to immune imbalance, particularly with impairment of T cell functions such as poor response to T cell mitogen, impaired GvH reaction, and lowered spleen T cell counts. Incidence and severity of amyloidosis induction in low dose (0.5 mg) thymosin –treated animals was reduced considerably, with a moderate improvement of spleen cell response to T cell mitogen. The increase in mitogenic responsiveness was higher when TF5 was given in high dose (2.5 mg) (Scheinberg, Goldstein and Cathcart, 1976). The effects of TF5 on disease resistance in protein deficient mice compared to healthy controls are opposite. Lysteria monocytogenes (LM) is associated with impaired T cell immunity. Protein malnourishment impairs LM resistance in mice. TF5 restored this resistance, but did not affect NK cell activity. In healthy animals however, TF5 reduced disease resistance and increased NK activity (Petro, Chien and Watson, 1982). Injections of high doses (0.1mg/day) of TF5 protected mice from streptozotocin-induced diabetes and hyperglycemia *in vivo*. Low dose (0.01 mg/day) TF5 did not have this protective effect. Additionally, the effects of thymosin on streptozotocin- induced diabetic rats also appear to vary depending on the maturational stage of T cells (Tabata et al., 1989). In contrast, TF5 is ineffective in the treatment of some disease conditions. For example, none of three different thymosin dose schedules moderated the incidence or severity of induced allergic encephalomyelitis in guinea pigs (Woyciechowska, Goldstein and Driscoll, 1985).

Thymosin facilitates murine host resistance to oncogenic viruses *in vivo*. Daily thymosin injections (4.0 mg) increased the percent of mice surviving more than twenty days after murine sarcoma virus- Maloney (MSV) infection to 69 %, compared to 8

percent for spleen extract-treated mice and none of control mice (Hardy et al., 1971). Splenocytes and thymocytes from Lewis tumor bearing mice enhance tumor growth in syngeneic animals. When TF5-treated thymocytes were used in this system, tumor growth was significantly decreased (Serrou et al., 1979).

As an anticancer agent, thymosin augmented the rejection of induced Dunning leukemia in rats. Effectiveness was dependent upon the time after disease induction that therapy started. The mean survival time for control animals was sixteen days, compared to greater than 365 days in thymosin treated animals, 60% of which experienced complete remission. Injection with normal lymphocytes also increased leukemia resistance. This effect was enhanced three fold when the lymphocytes used to treat experimental animals were obtained from control animals receiving thymosin injections. In contrast, rats receiving rabbit anti-thymocyte serum (ATS), an immunosuppressive agent believed to impair thymocyte function, were more susceptible to the disease. Thus, the effectiveness of thymosin in augmenting the anti-carcinogenic response may be the result of enhanced cellular immunity (Khaw et al., 1973).

Thymosin was also a successful adjunct for increasing tumor resistance of mice *in vivo*, synergistically increasing the effects of specific macrophage reactivity against subcutaneous murine MBL-2 or L1210 leukemia injection (Schultz et al., 1976). Moderate dietary protein deficiency increases *in vivo* resistance to L1210 murine leukemia cells as measured by lymphocyte mitogenic response *in vitro* and serum corticosterone levels, but not as measured by lymphocyte-mediated cytotoxicity. Protein deficiency in combination with TF5 administration *in vivo* enhanced this resistance as measured by all three parameters. TF5 alone also increased tumor resistance, but was not

correlated to any of these parameters (Petro et al., 1982). In contrast, administration of TF5 to adult thymectomized mice before and during injection with murine leukemia virus (MuLV) increased plasma virus titers, viral replication and the appearance of virus-induced membrane antigen, indicating that infection was enhanced (Forger et al., 1976).

Some of the conflicting reports of thymosin's efficacy as an anti- tumor agent may be a function of the tumor type under investigation. The injection of B16 melanoma cells into adult thymectomized irradiated mice results in lower incidence of metastasis compared to non-thymectomized control animals. In variants of this tumor, however, there is an increase in metastasis following thymectomy and irradiation. When the effects of thymosin were compared in both tumor types, it reversed the effects of thymectomy in both cases. That is, it increased the metastatic incidence resulting from B16 melanoma, and decreased it in the variant model. Thymosin did not affect metastasis in tumor bearing control animals. Thus, TF5 can act in lieu of an intact thymus (Naylor et al., 1983).

TF5 is effective as a primary treatment of some chemically induced murine tumors *in vivo*. Pre-incubation of syngeneic bone marrow cells in TF5, followed by intravenous administration into tumor bearing mice retarded tumor growth and increased survival time. This effect was not achieved with chemically synthesized compounds (levamisole, theophylline) that have thymosin-like activity *in vitro* (Cupissol et al., 1982). In the case of BBN induced tumor formation however, 60 μg of TF5 administered biweekly did not rescue male NON/shi mice from urinary tract carcinomas or metastasis of renal pelvic carcinomas (Murai et al., 1995). In contrast, TF5 is an effective adjuvant

combined with 5'-deoxy-5-fluorouridine (5'-dFUrd), enhancing its anti-tumor activity in mouse bladder cancer cells up to four fold (Ikemoto et al., 1999).

Because TF5 stimulates the hypothalamic-pituitary-adrenal (HPA) axis, our group also examined its effects on rat neuroendocrine MMQ pituitary adenoma and C6 glioma tumor cells *in vitro*. TF5 significantly reduces both the proliferative capacity and viability of both cell types. Morphological analyses indicate that apoptosis is the anti-proliferative mechanism. The effect on MMQ cells is partially reversed by MMQ- cell conditioned medium, indicating that these cells secrete an autocrine growth factor that opposes the actions of thymic hormones. None of the previously purified thymosin peptides (*i.e.* $T\alpha_1$, $T\beta_4$, MB-35 and MB-40) have any effect, so the observed inhibition may be due to a novel peptide component of TF5 (Spangelo et al., 1998). These results suggest that a thymic hormone contained in TF5 mediates neuroendocrine tumor formation and immune functions *in vitro* and may have therapeutic value in both capacities (Spangelo et al., 2000).

TF5 was tested *in vitro* as a differentiation agent for human leukemic T cells (MOLT-3). Incubation with TF5 markedly reduces the percentage of cells bearing surface markers of immature thymocytes (NAI/34) but does not affect expression of post-thymic T cell markers. Thus, TF5 induces signs of differentiation in human leukemic T cells *in vitro* (Ho et al., 1983). TF5 exerts similar effects in the human thymus-dependent leukemia (Thy-ALL) cell lines RPMI-8402 and JM1 (Ho et al., 1983a).

TF5: Clinical Studies

Clinically, TF5 has been used to replace thymus function in the treatment of human thymic disorders (*i.e.* Di George or Nezelof thymic hypoplasia, SCID, primary immunodeficiency), especially in cases of primary immunodeficiency, where thymus transplantation is problematic. It has also been employed extensively as an adjunct for enhancing immunity in "combined modality" studies, particularly in cases where the primary treatment regimen depletes or impairs the patients' immune system (as in chemotherapy). In some cases, however, TF5 has been a primary treatment, especially in autoimmune diseases, or for diseases for which no cure exists or other drugs have been ineffective (*i.e.* hepatitis).

In children with primary immunodeficiency, thymosin treatment increased cellular immune parameters both *in vitro* and *in vivo*. Weekly thymosin injections increased total lymphocytes and percentage of T cells in circulation, and improved reactivity to skin tests. *In vitro*, lymphocytes from treated patients formed more T cell rosettes, some responded better to mitogen stimulation and the MLR assay and some showed increased immunoglobulin and specific antibody synthesis. Thymosin caused partial reconstitution of cellular immunity in patients with thymic hypoplasia who have an existing population of thymocyte precursors, and induced an *in vitro* increase of T cell rosette formation by lymphocytes from patients with DiGeorge syndrome. In contrast, it had no effect *in vitro* or *in vivo* on the parameters for patients with SCID, severe combined immunodeficiency disease (Wara and Amman, 1976).

The immunorestorative potential of thymosin was tested clinically in an attempt to shorten the extended periods needed for marrow graft patients to recover

immunologically. Despite its success in restoring cellular immunity to lymphocytes from marrow transplant patients *in vitro*, TF5 did not significantly alter immune or disease stabilization parameters for human marrow graft recipients (Witherspoon et al., 1983).

TF5 is an effective immunorestorative adjunct in the treatment of some patients with advanced refractory cancer. In one clinical trial, a single treatment of thymosin resulted in significant improvement in several immunoassays designed to measure humoral and cell-mediated immune responses (*i.e.*, E-rosette, MLR). Although no decrease in tumor volumes was observed, two patients (14%), one with lung cancer and one with thymoma, had disease stabilization for up to six months. No significant toxicities, side effects, or complications resulted from thymosin therapy. Improvement in immune assays decreased by 48-72 hours, indicating that repeated thymosin administration is necessary to maintain benefits (Dillman et al., 1983). In contrast, sustained TF5 administration (120mg/mL biweekly) to patients with advanced colorectal cancer that had failed to respond to standard therapies, or patients with hypernephroma who had previously failed $T\alpha_1$ therapy, elicited no response in similar assays. Additionally, no antitumor effects were observed in this trial (Dillman et al., 1987). Similarly, administration of TF5 (60 mg/m^2 s.c. twice weekly) to small cell lung cancer patients undergoing induction chemotherapy and radiation therapy, including prophylactic cranial irradiation, did not alter response rate, response duration, survival rates, immune function, or lymphocyte counts (Scher et al., 1988).

Male homosexuals serum-positive for HTLV-III who entered therapy with abnormal T cell balances and received 60mg of subcutaneous TF5 daily for ten weeks exhibited improvements in T- lymphocyte immune (MLR) reactivity and transient

normalization of mitogen-induced IL-2 production. TF5 did not alter serum antibody titers, and other dose regimens (30, 120 mg) were ineffective. The responses could not be sustained when TF5 was administered only twice weekly. At a median 20-month follow up, none of the patients (6/14) whose MLR improved in response to TF5 had progressed to AIDS. These results indicate that TF5 has therapeutic value for the treatment of immune deficiencies in serum-positive HTLV-III patients in appropriate doses and in combination with antiviral drugs (Schulof, et al., 1986).

Several autoimmune disorders possibly related to abnormal T suppressor functions responded well to TF5 treatment in initial clinical studies. Four patients with systemic lupus erythematosus, and one with rheumatoid arthritis and Sjogren's syndrome were given thymosin injections for 2 to 35 months. All patients had higher levels of T lymphocytes in circulation after treatment. Pretreatment assays were positive for cytotoxicity for all patients, and declined to zero during the course of therapy. Of four patients exhibiting clinical improvement, one had disease stabilization (Lavastida et al., 1981).

Preliminary clinical results indicate that thymosin is effective as a primary treatment for chronic hepatitis B. Thymosin treated patients were compared to patients receiving placebo, and both groups were compared to healthy volunteers. Six of seven thymosin-treated patients responded to therapy. One of the five placebo-treated patients experienced complete remission of disease, while the other four showed no response. In thymosin patients, serum HBV DNA disappeared and serum ALT levels were significantly lower than placebo patients. The thymosin group was negative for serum HBV DNA and had normal ALT levels at two years post treatment. Thymosin also

reversed the decrease in peripheral blood lymphocyte, CD4 and CD3 counts caused by the disease, and these patients had elevated levels of IFN-γ synthesis by peripheral blood mononuclear cells (PBMC) as compared to the placebo group and to healthy volunteers. Thymosin therapy was also associated with decreased liver inflammation and tissue damage, and cessation of viral protein production (HbsAg) and HBV DNA replication. Since the thymosins are not known to have antiviral properties *in vivo*, these clinical results may be due to thymic peptide modulation of cellular and humoral immune responses (Mutchnik et al., 1991).

Patients undergoing intensive chemotherapy to induce remission of small-cell lung cancer did not exhibit higher complete response rates upon the addition of thymosin therapy (60 mg/m^2 biweekly for six weeks). However, among the complete responders, those who received thymosin had prolonged disease remission and subsequently, significantly prolonged survival times (Cohen et al., 1979).

In a clinical trial evaluating the efficacy of TF5 as a primary therapy in advanced renal cancer, thymosin treatment of patients who had previous nephrectomy resulted in partial regression in 15%, and disease stabilization in 10% of patients. All patients who responded to therapy presented with lung metastases prior to treatment. One patient had a 50% reduction in diameter of a lung lesion, accompanied by complete clearing of a small pleural effusion, after four weeks of high dose (120mg/m2) thymosin administration. In this trial TF5 was as effective as chemotherapy in the treatment of advanced renal cancer, without the detrimental side effects. These results also suggest that thymosin may be effective in the therapy of lung cancer patients (Schulof et al., 1984).

Thus, TF5 and its component peptides participate extensively in immune functions, and can improve host defense by stimulating both cellular and humoral immunity. Additionally, they can directly inhibit disease and tumor progression. Their effects vary depending on the experimental model system and on the type of disease or cancer involved. Their effectiveness for a particular function *in vitro* may be an indicator of therapeutic usefulness.

Principle Hypothesis of This Study

Because TF5 is a potent immune enhancing agent that also exerts anti-cancer effects, and because the leukemias are cancers that induce severe immune deficiency, we hypothesized that TF5 would act directly on human leukemia cells to inhibit their proliferation. We conducted experiments to test the specific hypotheses that (1) thymic peptides inhibit the proliferation of human HL-60 myeloid leukemia cells, (2) this inhibition is due to a putative peptide component of TF5, and (3) the mechanism of inhibition is apoptosis.

Leukemia and the Origins of the HL-60 Cell line

The leukemias are cancers that result from the unregulated growth and abnormal differentiation of hematopoietic cells and their precursors (stem cells) in the bone marrow. The leukemic cells proliferate more and survive longer than normal cells, gradually building up in the bone marrow and inhibiting the production of functional blood cells. Eventually, aberrant nonfunctional leukocytes spill into the bloodstream and ultimately invade other organs and tissues, thwarting normal function. Impaired

hematopoiesis leads to deficiencies of red blood cells (anemia), platelets (thrombocytopenia) and phagocytes (neutropenia, monocytopenia), and disrupts the ability to mount a competent immune response.

There are four major classifications of leukemia: lymphocytic leukemias involve abnormal development of lymphocytes, while myelogenous (granulocytic) leukemias affect cells of the myelocytic lineage. Both of these may be acute or chronic. Acute leukemias progress very rapidly; chronic disease progresses more slowly then converts to rapid progression or "blast crisis" in the terminal stage.

Acute myelogenous leukemia (AML), also known as acute non-lymphocytic leukemia (ANLL) or acute granulocytic leukemia (AGL), results from neoplastic transformation of a hematopoietic stem cell in the bone marrow. The cause of transformation is generally not known, but several risk factors have been identified. These include advancing age (~50% of new cases occur after age 65), impaired immune function, exposure to industrial chemicals (*i.e.* benzene), or exposure to irradiation. Paradoxically, chemotherapy, the primary treatment for AML, is also a major risk factor for the disease. In fact, AML commonly occurs as a secondary malignancy in patients previously treated for cancer (Belcher, 1993; McAllister, Horowitz and Gilden, 1993).

Chemotherapy also contributes to another risk factor for leukemia, impaired immune function. It is unclear whether immunodeficiency allows leukemia to develop or whether the neoplasm and the immune defect arise from the same genetic injury. Several congenital immunodeficiencies however, are associated with increased AML incidence (*i.e.* SCID, neutropenia, Fanconi's anemia), and the course of AML itself results in severe immune defects (*i.e.* neutropenia, anemia, thrombocytopenia). Without competent

immune defense, patients are increasingly susceptible to infections (Wiernik et al., 1991; Belcher, 1993; McAllister, Horowitz and Gilden, 1993). Impaired immunity is also associated with poor prognosis, especially if T cell dependent immunity is affected, as exemplified in the case of Hodgkin's disease. Hodgkin's disease is a neoplasm affecting the lymph nodes, spleen and liver. Otherwise normal patients with Hodgkin's disease have a 66% higher 5-year survival rate then patients with deficiencies involving cell-mediated immunity (Wiernik et al., 1991). Because of these factors, there is an emphasis on restoring and maintaining immune functions during AML therapy.

The HL-60 human acute myelogenous leukemia cell line was established from the peripheral blood of a patient with AML (Collins, Gallo and Gallagher, 1977). It was originally classified as derived from acute promyelocytic leukemia APL FAB- M3, a sub-type of the myelogenous leukemias, although the cells were noted to lack several features characteristic in APL cells (Collins et al., 1977; Gallagher et al., 1979). It was later reported that HL-60 morphology is more accurately classified as AML FAB- M2, an acute myeloblastic leukemia with some markers of maturation (Dalton et al., 1988).

This cell line is susceptible to HIV-1 and HTLV-1 viruses. The cells form colonies in agar and produce myeloid tumors in nude mice. HL-60 cells are commonly used for pharmacodynamics, induction of differentiation studies and antitumor testing (Gallagher et al., 1979; Koeffler and Golde, 1980).

Since TF5 generally restores immune deficiencies and inhibits the activity of some tumor cells, we tested its effects on human myelogenous leukemia cells.

CHAPTER 2

MATERIALS AND METHODS

Chemicals and Reagents

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, z-Val-Ala-Asp-CH_2F (zVAD-fmk), etoposide (VP-16), sphingomyelinase, horse heart cytochrome c, trypsin, bradykinin, aprotinin, sephadex G-50 gel filtration beads, reagent grade isopropanol, methanol, glacial acetic acid, hydrochloric acid, dibasic sodium phosphate, ammonium sulphate, β-mercaptoethanol, Folins reagent and agar were purchased from Sigma Chemical Company (St. Louis, MO). Dr. Ronald Gary, University of Nevada Las Vegas (Las Vegas, NV) kindly provided DNP-Asp Blue Dextran and Coomassie blue stain solution. Penicillin G-streptomycin sulfate antibiotic mixture, streptomycin sulfate, RPMI 1640, L- Glutamine, MEM sodium pyruvate solution, MEM non-essential amino acids solution, and trypan blue stain were purchased from Gibco BRL (Grand Island, NY). Heat-inactivated Fetal Clone I serum was purchased from HyClone, USA (Logan, UT). Dr. Mahnaz Badamchian, George Washington University (Washington, DC) generously provided thymosin fraction 5 (TF5) and thymus acetone powder (TAP). Dr. W. David Jarvis, University of Texas at Houston (Houston, TX) kindly provided the HL-60 cell line. The CRL7686 cell line was obtained from the American Type Tissue Culture

Collection (Rockville, MD). Novex™ Tris-glycine 4-20% SDS (sodium dodecyl sulfate) and NuPAGE™ Bis-tris 12% pre-cast electrophoresis gels, Novex™ precast vertical IEF gels, Mark 12™ electrophoresis protein standard mixture, tris-glycine SDS running buffer, tris-glycine SDS sample buffer, NuPage MES (2-(N-morpholino) ethane sulfonic acid) SDS running buffer, IEF anode buffer, IEF cathode buffer pH 3-10 and pH 3-7, IEF sample buffers pH 3-10 and pH 3-7, and colloidal blue staining kit were purchased from Invitrogen Corporation (Carlsbad, CA).

HL-60 Cell Culture

The HL-60 cell line was maintained in continuous culture in a humidified atmosphere of 5% CO_2-95% air at 37°C in phenol-free RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum, sodium pyruvate, nonessential amino acids and antibiotics. Cells were sub-cultured every 3-4 days. Cells were removed from tissue culture flasks, rinsed three times with serum- free RPMI (sfRPMI), counted by hemacytometer, and either placed in continuous culture (1.0×10^6 cells per 25 cm^2 flask) or used in cell culture assays.

Viable Dye Exclusion Assay

Initial experiments consisted of three groups of cells: one was pre-incubated in the presence of TF5 for 24 h and then rinsed and assayed, a second was exposed only to vehicle (RPMI 1640, supplemented), and a third was assayed in the continuous presence of TF5. All groups were cultured at an initial plating density of 1.0×10^6 cells/flask in 25 cm^2 tissue culture flasks and aliquots were removed and counted by hemacytometer each

day for four days using the viable dye (trypan blue) exclusion assay. Cells were also cultured in 12-well culture plates (240 x 10^3 cells/3ml/well; Costar, Fisher Scientific, Santa Clara, CA) and treated with vehicle or TF5 for 24-96 h for the determination of cell viability and proliferation using the viable dye exclusion assay. For the TF5 metabolism assay, cells were cultured in 25 cm^2 tissue culture flasks (1.0 x 10^6 cells/flask; Greiner Labortechnik, ISC Bioexpress, Kaysville, UT) in the presence of either TF5, HL-60 conditioned medium, HL-60-conditioned TF5 or vehicle, and aliquots were removed and counted by hemacytometer each day for four days

For the MTT assays, HL-60 cells were incubated in 96-well plates for 72 h (20 x 10^3 cells/0.2 ml/well; Greiner) in the presence of either TF5, fractions of TF5, other thymus preparations, or vehicle for the determination of cell viability and proliferation. In addition, differing numbers of cells (5-30 x10^3 cells/well) cultured in 96-well tissue culture plates were exposed to TF5 for 96 h. After incubation, 20µl of 5mg/mL MTT was added to each well. After a 4 h incubation period, 150µl was removed from each well, and replaced with an equal volume of 0.04 M HCl/isopropanol. After overnight incubation in darkness, the dissolved MTT crystals were quantitated. Optical densities were obtained using a test wavelength of 570 nm and a reference wavelength of 630 nm (Dynatech MR5000 microelisa spectrophotometer, Chantilly, VA).

Clonogenic Assay

Cell proliferation was also evaluated using a cloning assay. HL-60 cells were removed from continuous culture, rinsed three times with sfRPMI and counted by hemacytometer. Various cell numbers (50-1000 cells/well) were dispersed into 12-well

plates in a single-layer soft agar system containing RPMI, 20% heat- inactivated fetal bovine serum, and 0.3% agar. The plates were incubated at 37°C in 5% CO_2-95% air. The resulting colonies (>50 cells) were counted 14 days after plating. In TF5 pretreatment experiments, cells were removed from continuous culture, rinsed with sfRPMI and 1.0 x 10^6 cells/10mL were incubated in culture flasks either in vehicle, or in 100 - 800 μg/mL TF5 for 24 h. They were then rinsed, counted and plated in the agar system for enumeration of colony formation after 14 days of incubation. Using the same protocol, cells were also incubated for 24h with vehicle, 400 and 800 μg/mL TF5, or 40 and 80 μg/mL of biologically active RP-HPLC fractions of TF5 and plated in the agar system. In time-course experiments, 1.0 x 10^6 cells/10mL were incubated in culture flasks either in vehicle, or in 400 and 800 μg/mL TF5 for 3-24 h. The end points of incubation were coordinated so that all cells were removed from culture or treatment, rinsed and plated in the agar system at the same time. To study the effects of apoptosis inhibitors, cells were removed from culture as before, and one group was pretreated for 30 min with the apoptosis inhibitor ZVAD-fmk (dissolved in DMSO), and then incubated in the presence of 800mg/mL of TF5. Other groups were incubated with vehicle, DMSO (as a control for the ZVAD-fmk carrier) or 800 μg/mL TF5. All groups were plated in the agar system at the same time. In similar experiments, cells were pre-incubated with ZVAD-fmk for 30 min and then incubated with sphingomyelinase (Smase), or etoposide or 800μg/mL TF5, or cells were incubated with 800μg/mL TF5, DMSO, or vehicle for 24 h, and then plated in the agar system. As before, incubation times were coordinated so that cells were rinsed and plated at the same time.

Partial Purification of TF5

TF5 was prepared from freshly frozen calf thymus as described by Hooper et al (1975). Preparative reverse-phase high performance liquid chromatography (rpHPLC) of TF5 was performed as described as described by Badamchian, et al (1990, 1991). TF5 (1.5 g) was applied to a δ-prep HPLC system equipped with a model 481 variable wavelength detector with a semi-preparative flow cell (280 nm) and a 300 x 50 mm d-pak 300 Å 15μm C_{18} column (Waters Chromatography Division of Millipore Corp., Milford, MA). Eluent A was 0.02 M ammonium acetate (pH 6.8) and eluent B was acetonitrile. A 60-min linear gradient from 0-80 % B was run at a flow rate of 80mL/min. TF5 was dissolved in the initial solvent A, applied to the column through a port in the solvent delivery system, and one- minute fractions were collected for sixty minutes. Actual protein concentrations were determined using the Lowry assay (Lowry et al., 1951). Aliquots from each of the 60 fractions (5-15μl/well) were added to cultured tumor cells. The cells were incubated for 4 days, and the extent of proliferation was determined using the MTT assay.

Size-Exclusion HPLC Analysis of TF5

TF5 was further separated by size-exclusion HPLC. 20 or 50μL of TF5 dissolved in 10mM dibasic sodium phosphate buffer was injected onto an in-line column system composed of a Macrosphere GPC 100A 7μ column 250 mm x 4.6 mm (i.d.) followed by a Macrosphere GPC 60A 7μ column of the same dimensions (Alltech Associates, Deerfield, IL), with a 10 mM dibasic sodium phosphate running buffer. Detection was obtained at 220nm (Spectroflow 757 absorbance detector, Kratos, Ramsey, NJ). The

resulting protein peaks were analyzed by PeakSimple PeakII Chromatography Data System software (SRI Instruments, Torrance CA.). The fractions collected were lyophilized under vacuum in centrifugal desiccation unit (Savant Instruments, Inc., Holbrook, NY), and re-suspended in sterile ultrapure water for further analysis.

Gel filtration Chromatography

Purification of TF5 components was achieved by gel filtration chromatography. A Kontes (Fisher Scientific, Santa Clara, CA) 1.5 x 30 cm (50 mL) filtration column was packed with Sephadex G-50 beads in 10mM sodium phosphate buffer adjusted to pH 7.0. The flow rate was maintained at 1.40 mL/min. The outer and inner column volumes were defined as the elution volumes of blue dextran (5 mg/mL) and DNP-Asp (0.5 mg/mL) respectively. Bradykinin MW ~1050), cytochrome c (MW ~12,000), and trypsin (MW ~24,000) were used as protein standards. From their elution volumes, the elution coefficient, K was determined, using the following equation:

$$K = (V_e - V_o)/(V_t - V_o)$$

Where V_e is the elution volume of a solute, V_o is the volume outside of the beads in the column, and V_t is the total volume occupied by the gel bed.

A plot of the log of the molecular weight versus the K for the standard proteins was used to estimate the molecular weight of the TF5 fractions. TF5 dissolved in 10mM sodium phosphate was applied to the column and the resulting fractions were collected and stored at -70°C. They were later lyophilized under vacuum in centrifugal desiccation unit (Speedvac SC100, Savant Instruments, Inc., Holbrook, NY), and re-suspended in sterile ultrapure water for further analysis.

Electrophoresis

SDS PAGE electrophoresis (Laemmli) and isoelectric focusing were carried out in a mini-vertical electrophoresis system (Novex Xcell Surelock Mini-Cell, Invitrogen) on pre-cast 10- well gels in accordance with the manufacturers protocols (Invitrogen-Novex™). Initial SDS PAGE gels were 4-20% tris-glycine polyacrylamide gels buffered to pH 8.7 with a tris-glycine-SDS (2.9%, 14.4%, 1%) running buffer (upper and lower chambers) pH adjusted to 8.3. The sample buffer was composed of 0.5 M Tris-HCl, 20% glycerol, 10% (w/v) SDS, 0.1% bromophenol blue, pH adjusted to 6.8. The samples were reduced by adding 2.5% β-mercaptoethanol and heating at 85° C for two minutes immediately prior to loading. 5-50 μg of sample was loaded into each well, and electrophoresis was carried out at 25 mA starting and 8 mA ending current (125V constant) for approximately 60 minutes. The gels were subsequently stained in a solution containing 0.06% Coomassie Blue, 50% methanol, and 7% glacial acetic acid in ultrapure water for approximately 12 hours, and then de-stained for approximately 16 hours in a destain solution containing 10% methanol and 7% glacial acetic acid in ultrapure water. Gels were then permanently fixed on a slab gel drier (SGD 4050 Slab Gel Dryer, Savant Instruments, Inc., Holbrook, NY).

Electrophoresis was also carried out on 10-well 12% Bis-Tris-HCl (Bis[2-hydroxyethyl]imino-tris[hydroxymethyl]methane-HCl) buffered (pH 6.4) polyacrylamide gels (NuPage ™Bis-Tris gels) in accordance with the manufacturer's protocol (Invitrogen-Nupage). The lower chamber running buffer was composed of 1.00 M MES, 1.00 M Tris base, 69.3 mM SDS, and 20.5 mM EDTA (ethylenediaminetetraacetic acid). The composition of the upper (inner) buffer chamber running buffer was the same as for

the lower, with the addition of 0.25% of Nupage™ antioxidant (Invitrogen) containing dimethylformamide and sodium bisulfite. The sample buffer (pH 8.5) was composed of 40% glycerol, 6.82% tris base, 6.66% tris HCl, 8% LDS (lithium dodecyl sulfate), 0.06% EDTA, 0.075% Serva Blue G250 stain, and 0.025% phenol red. Two gels were run simultaneously. 3-5μg of sample was loaded onto each well, and electrophoresis was carried out at 200 V constant voltage for approximately 30 minutes. Gels were then shaken in fixative consisting of 50% methanol, 10% glacial acetic acid and 40% ultrapure water for 10 minutes, stained in colloidal blue stain solution and destained in ultrapure water according to the protocol provided by the manufacturer (NuPage ™ Colloidal Blue Stain Kit-Invitrogen). Gels were stored in 20% ammonium sulfate solution at 4 °C until drying.

Cytological Analysis

Cytological characterization of apoptosis was performed by Jarvis and colleagues as previously described (1997). To examine cells for the apoptotic morphological changes, they were stained with 20% Wright-Giemsa stain and assessed for the expression of nuclear and cytoplasmic condensation, membrane blebs, apoptotic nuclear bodies and cell shrinkage by light microscopy (LM). At least four 50-cell fields were scored.

To detect apoptotic DNA damage, cells that were prepared and fixed as described (Jarvis et al., 1997) were stained to detect broken DNA by treatment with TdT in the presence of fluorescein isothiocyanate-dUTP (FITC-dUTP) and then counterstained for intact DNA with propidium iodide. At least four 50- cell fields were scored by

fluorescence microscopy (FM) by evaluation of increased direct fluorescence of end-labeled double-stranded DNA.

Statistical Analysis

Analysis of variance (ANOVA) and the Bonferroni analysis for multiple comparisons were used for statistical evaluation of the data. A *P*-value of ≤ 0.05 was considered significant. Unless noted otherwise, data are expressed as the mean $\pm$ SEM of groups consisting of 4 observations. Unless otherwise noted, each experiment was performed independently at least 3 times and representative results are reported.

CHAPTER 3

RESULTS

Previously reported results using MMQ (rat pituitary adenoma) and C6 (rat glioma) cells determined that TF5 is an effective inhibitor of proliferation in neuroendocrine tumor cells (Spangelo et al., 1998, 2000). Because TF5 exhibited these anti-proliferative abilities on MMQ and C6 cells, and because it is a known stimulator of T cell-mediated immunity, we examined its capabilities as an inhibitor of human leukemia cell proliferation.

Continuous Culture of HL-60 cells

In continuous culture, HL-60 cells proliferated rapidly, doubling in concentration (cells/mL) every 24-30 hours (Fig 4). Cell viabilities consistently remained above 95% during the culture period (data not shown). Cells were removed from culture and assayed during the logarithmic phase of proliferation that occurred at 24-96 hours.

Effects of TF5 on HL-60 Cell Proliferation: Dye Exclusion and MTT Assays

TF5 (100-800 μg/mL) suppressed the proliferation of HL-60 cells during 24-72 hours of exposure (Fig 5), with cell viability being reduced to 60% within 48 hours as

determined by the viable dye exclusion assay (Table 1). At 400 μg/mL TF5, cell proliferation was inhibited by 50% (ED_{50}) and 800 μg/mL TF5 consistently achieved maximal inhibition. This anti-proliferative effect increased with time, demonstrating maximal inhibition at 72 hours of incubation (Fig 6).

In the MTT assay, TF5 was less effective as an anti-proliferative agent at higher cell densities (Fig 7). While all concentrations of TF5 inhibited proliferation at initial plating densities of 5 and 10 x 10^3 cells per well, higher concentrations of TF5 were required to significantly inhibit cell proliferation at greater cell densities. An initial plating density of $20x10^3$ cells/well allowed was chosen for all subsequent MTT reaction assays. This initial density allows a clear TF5 dose-response with an ED_{50} at approximately 400 μg/mL and maximal inhibition at 800 μg/mL, consistent with the viable dye exclusion assay.

To examine the mechanism by which inhibition was effected, we tested the ability of HL-60 cells to rebound from 24 hours of pretreatment with 800 μg/mL of TF5 as measured by the viable dye exclusion assay. Cells that had been pretreated with TF5 and then rinsed were re-cultured in vehicle and compared to cells cultured only in vehicle, and cells cultured in vehicle with 800 μg/mL of TF5. Pretreatment with TF5 (24 hours) produced the same capacity for suppressed proliferation as continual treatment (Fig 8). These results suggested that TF5 induced its anti-proliferative effects within 24 hours. Its removal did not diminish the observed inhibition. Because of this, we hypothesized that the cells metabolize a consumable factor found in TF5. To test this hypothesis, the supernatant from cells treated with 800 μg/mL TF5 for 24 hours was applied as a conditioned medium to a naive cell culture (HL-60-conditioned TF5). This was compared

to cells cultured in vehicle, 800 μg/mL TF5, and 800 μg/mL TF5 dissolved in HL-60 conditioned medium. The proliferation of cells treated with TF5-conditioned medium was similar to that of cells cultured in vehicle. In contrast, the effects of treatment with 800 μg/mL TF5 were the same in both vehicle and HL-60-conditioned medium (Fig 9). These preliminary results support our hypothesis.

Effects of TF5 on HL-60 Cell Proliferation:

Clonogenic Assay

Cell proliferation can also be measured using a single layer soft agar system to determine the capacity of the cells to form colonies. In contrast to the viable dye exclusion and MTT assays, clonogenic potential is a direct measure of cell viability and proliferative capacity. Therefore, the clonogenic potential of HL-60 cells was tested in the presence and absence of TF5. Initial experiments demonstrated that the clonogenic potential of HL-60 cells was dependent on initial cell number (Fig 10). We chose 300 cells/well as the initial cell plating density in all subsequent experiments because this cell concentration allowed concentration-dependent effects of TF5 similar to those observed in the viable dye exclusion and MTT assays.

The ability of TF5 to inhibit the clonogenic potential of HL-60 cells was tested. Cells were pretreated with concentrations of TF5 ranging from 100-800 μg/mL. TF5 reduced the ability of HL-60 cells to form colonies in a dose dependent manner. The dose producing 50% inhibition (ED_{50}) was 400 μg/mL and 800 μg/mL was maximally effective, as with the MTT assay (Fig 11). To determine the minimum incubation period required for inhibition of colony-forming potential, pretreatment time course experiments

were conducted. Exposure to 400 μg/mL for as little as 4 hours produced inhibition of colony formation. Exposure to 800 μg/mL of TF5 for 4 hours significantly inhibited colony formation, and 8 hours essentially completely inhibited HL-60 clonogenic potential (Fig 12).

Effect of zVAD-fmk on TF5 Inhibition of HL-60 Cell Proliferation

The mechanism of inhibition was investigated using the known apoptosis inhibitor, z-Val-Ala-Asp-CH_2F (zVAD-fmk), a cell-permeable, broad-spectrum irreversible caspase inhibitor. We compared the ability of zVAD-fmk to reverse the effects of TF5 to its ability to reverse the effects of etoposide, a known apoptosis inducer. In these experiments, zVAD –fmk was not able to reverse the effects on HL-60 cells of either TF5 or etoposide (data not shown). Since it has been recently reported that zVAD-fmk does not universally protect cells from apoptosis, (Zhang, Sheng and Loh, 1999; Amarente-Mendes et al., 1998), these experiments were repeated using another known apoptosis inducer that acts through a different mechanism, sphingomyelinase (Smase) (Fig 13). Again neither the effects of TF5 nor of Smase were reversed by zVAD-fmk. Therefore, cytological analyses were performed to determine whether TF5 treatment induces the appearance of morphological and cytoarchitectural features of apoptosis in HL-60 cells.

Effects of TF5 on HL-60 Cell Proliferation:

Cytological Analysis

Cells were treated with vehicle or excessive doses of known apoptosis inducers (ceramide, sphingosine) or maximal doses of TF5 and then examined for membrane blebbing, nuclear and chromatin condensation, apoptotic nuclear bodies and cell shrinkage by light microscopy (LM). The terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was used to assess by fluorescence microscopy (FM) the induction of apoptotic DNA damage in the cells. The cytological effects of lipid messengers known to induce apoptosis (Jarvis et al., 1997) are presented in Table 2. These compounds caused the appearance in HL-60 cells of cytological features (*i.e.* membrane blebbing, DNA damage) characteristic of apoptosis. In contrast, the data presented in Table 3 shows that treatment with maximal doses of TF5 did not induce cytological features associated with apoptosis. These experiments confirm that apoptosis is not involved. Rather, TF5 appears to induce cytostasis in HL-60 cells.

HPLC Analysis of TF5: Reverse Phase

TF5 can be separated on isoelectric focusing gels into several components. Three peptide components, $T\alpha_1$, $T\beta_4$, and MB-35 have been purified and sequenced. None of these purified thymosin peptides ($T\alpha_1$, $T\beta_4$, MB-35) had any effect on HL-60 cells in pharmacological doses (Fig 14). Therefore, TF5 was further purified using reverse- phase HPLC (rpHPLC). Of the sixty resulting fractions, only fraction 24 demonstrated consistent inhibition of HL-60 cell proliferation (Fig 15). As an anti-proliferative agent, fraction 24 was ten times more potent than TF5 (Fig 16).

HPLC Analysis of TF5: Size Exclusion

Due to the limited amount of sample produced by rpHPLC we were unable to obtain sufficient quantities for thorough analysis and purification of TF5-fraction 24. Therefore, TF5 was also separated by size exclusion gel permeation HPLC. Because TF5 contains a number of small peptides, a standard curve was generated using proteins in the molecular weight range of 1050 Da to 66 kDa (Fig 17). None of the resulting TF5 fractions consistently demonstrated significant inhibition of HL-60 cell proliferation (Fig 18).

Gel Filtration Chromatography Analysis of TF5

To more effectively purify useful quantities of its anti-proliferative activity, TF5 was separated on the basis of molecular size by gel filtration chromatography on a Sephadex G-50 gel bed with a flow rate of 1.4 mL/min. The standard curve for protein standards in the molecular weight range 1060 Da to 24 kDa is shown in Fig 19. According to this standard curve, the most active TF5 fraction (fraction 5) corresponds to approximately 20 kDa molecular weight. Since TF5 contains only peptides of less than 15 kDa, it is possible that oligomerization of component peptides may occur in the biologically active form of the extract. In Fig 20, the protein concentrations are compared to the anti-proliferative activity of the fractions resulting from the separation of TF5 (50 mg) by gel filtration chromatography.

Gel Electrophoresis of TF5 and Biologically Active TF5 Fractions

Molecular weight estimates of the TF5 fractions were performed under reducing and denaturing conditions using polyacrylamide gel electrophoresis. Biologically active TF5 gel filtration chromatography fractions were separated on Tris- glycine SDS- PAGE gels. Mobilities were compared to those of wide- range molecular weight protein standards (2.5 kDa – 200 kDa) shown in Fig 21. The behavior of TF5 and two less purified thymus extract preparations (TP12900586, TP2169066) are also shown. The most predominant band of the active fraction on these gels corresponds to a molecular weight estimate of less than 14 kDa (Fig 22). Bis- Tris gels were used to achieve better separation of very low molecular weight species. Mobilities were compared to those of wide- range molecular weight protein standards (2.5 kDa – 200 kDa). The standard curve for these gels is shown in Fig 23. Fig 24 shows that the TF5 fractions are still heterogeneous, and further purification is required before sequencing can be attempted. Four predominant bands are present in the molecular weight range 5000-15000.

Effects of Other Thymic Extract Preparations On HL-60 Cell Proliferation: Comparison to TF5

To determine whether the anti- tumor activity present in TF5 can be isolated from other thymus extracts, its effects were compared to those of less purified bovine thymus extract preparations. Thymus preparations (TP, TAP) inhibited the proliferation of HL-60 cells in a manner similar to that of TF5 (Fig 25). Reverse-phase HPLC fractions of one

thymus acetone preparation, TAP, contain both inhibitory and stimulatory factors (Fig 26). The Lowry protein profile of TAP fractions indicates that the area of greatest inhibition of tumor cell proliferation corresponds to the protein peak, whereas the highly stimulatory activity precedes the protein peak. TAP fraction 20 inhibited HL-60 cell proliferation by as much as 80%, whereas fraction 10 induced substantial proliferation in the cells. A dose response comparing the potency of TAP fraction 20 in HL-60 cell inhibition to that of TF5 and the parent extract (TAP) is shown in Fig 27. As with TF5, further separation of the anti-proliferative activity by size-exclusion HPLC was unsuccessful (data not shown).

CHAPTER 4

DISCUSSION

Thymosin fraction 5 is a partially purified protein extract of the bovine thymus that contains at least 30-40 heat-stable polypeptides. It is frequently used as a replacement for an intact thymus in various *in vitro* and *in vivo* models. TF5 stimulates multiple immune functions and exhibits anticancer effects, making it attractive as a potential source of medications to treat immune disorders and cancers. We have previously reported (Spangelo et al., 1998) that TF5 inhibits the proliferation of the rat pituitary adenoma MMQ and rat C6 glioma cell lines *in vitro*, but induces only a modest suppression of proliferation in the CRL7686 human melanoma cell line. TF5 has also been shown to thwart the proliferation of certain murine leukemia cells (Khaw et al., 1973; Schultz et al., 1976; Petro et al., 1982) and human acute lymphoblastic T cell leukemia cells *in vitro* (Ho et al., 1983, 1983a). Furthermore, TF5 normalized the *in vitro* immune responses of human T cells from leukemia patients (ALL, CLL, AML) (Schafer, et al., 1976).

The *in vitro* effects of TF5 on human acute myelogenous leukemia cell proliferation have not been tested, however, and the presumed activity in TF5 that is responsible for the inhibition of tumor cell proliferation has not been identified. In addition, the mechanism of TF5 inhibition of myelogenous leukemia cells has not been

determined. Therefore, we studied the effects of TF5 on HL-60 human acute myelogenous leukemia cells.

HL-60 Cell Culture

The HL-60 cell line responded well in continuous suspension culture under the conditions described, maintaining high cell viability (>90%) throughout the culture period. The cell-doubling time was 24-30 hours during the logarithmic growth phase. HL-60 cells also formed colonies spontaneously in soft agar in a manner that was dependent on initial cell plating density.

Effects of TF5 on HL-60 Cell Proliferation:

Viable Dye Exclusion and MTT Assays

We found that TF5 inhibited the proliferation of HL-60 cells *in vitro* in a dose, time and cell number- dependent manner. As with the MMQ and C6 neuroendocrine tumor cell lines (Spangelo et al., 1998), the suppression of HL-60 proliferation was accompanied by only a modest reduction in cell viability. The TF5 ED_{50} for HL-60 cell growth suppression was approximately 400 μg/mL, and maximal inhibition (≤ 20% of control cultures) was achieved with a concentration of 800 μg/mL at 72 hours of incubation. In contrast, 1000 μg/mL of TF5 only moderately suppressed CRL7686 human melanoma cell proliferation (60% of control cultures at 1000 μg/mL), with an ED_{50} of approximately 700 μg/mL. As with the MMQ, C6 and CRL7686 cells, none of the purified thymosin peptides ($T\alpha_1$, $T\beta_4$, MB-35) had any effect on the proliferation of the HL-60 cells. Together, these results indicate that TF5 may not exert a non-specific effect in the inhibition of HL-60 cell proliferation.

TF5 had less effect on HL-60 proliferation at higher initial cell densities. Depending on the cell density, concentrations as low as 100 μg/mL significantly suppressed HL-60 cell growth. However, concentrations below 600 μg/mL did not block the proliferation at an initial plating density of $30x10^3$ cells/well. The effects of low concentrations of TF5 were also increasingly less dramatic with time, suggesting that an anti-TF5 activity accumulates in the HL-60 cell cultures. Similar effects were observed in the MMQ cell line, and we found that 50% MMQ-conditioned medium largely reversed the TF5 inhibition of MMQ cells (Spangelo et al., 1998), supporting the hypothesis that MMQ cells release a factor that interferes with TF5 inhibition of cell growth. However, in characterizing the HL-60 cell line, Gallagher and colleagues (1979) found that HL-60-conditioned medium does not stimulate the *in vitro* proliferation or clonogenic potential of normal bone marrow cells or leukemic leukocytes. In a later study, this group analyzed the ability of HL-60 conditioned medium and concentrated HL-60 cell extracts to stimulate their own clonogenic potential. None of the HL-60 conditioned preparations were able to stimulate the colony formation of HL-60 cells or normal bone marrow cells (Ruscetti et al., 1981). This indicates that HL-60 cells may not release an autonomous growth factor that interferes with the action of TF5.

Additionally, we found that HL-60 cell counts and MTT-derived optical density (OD) units were significantly reduced within 24 hours in TF5 treated cultures as compared with control cells. TF5 suppression of HL-60 cell proliferation was not diminished by the removal of TF5 after the initial incubation period. Further, when TF5 in medium was exposed to HL-60 cells for 24 hours, it was not able to inhibit the

proliferation of naïve cell cultures. It appears that the HL-60 cells metabolize a consumable factor found in TF5.

Effect of TF5 on HL-60 Cell Proliferation:

Clonogenic Assays

In semi-solid medium such as agar, HL-60 cells form spontaneous colonies without the necessity for exogenous growth factors (Gallagher et al., 1979; Ruscetti et al., 1981). TF5 inhibited the cloning potential of HL-60 cells in a dose-dependent manner, suggesting that an activity in TF5 prevents the occurrence of HL-60 colony formation in agar. As with the MTT and viable dye exclusion assays, the TF5 ED_{50} for the inhibition of colony formation was approximately 400 μg/mL, and maximal inhibition was achieved with a concentration of 800 μg/mL. Exposure to 400 μg/mL of TF5 for as little as 4 hours retarded colony formation and 800 μg/mL of TF5 for 8 hours was sufficient to completely inhibit HL-60 clonogenic potential. We suggest that a thymic peptide may prevent metastatic growth of human leukemia cells *in situ*. The results of the clonogenic assay are consistent with those obtained using the MTT and trypan blue exclusion assays, both of which demonstrated a decrease in cell viability and proliferation in the presence of TF5.

Mechanism of TF5 Inhibition of

HL-60 Cell Proliferation

We used known inducers (etoposide, sphingomeylinase) and inhibitors (zVAD-fmk) of apoptosis to study the mechanism of this inhibition. The broad-spectrum caspase inhibitor zVAD-fmk failed to reverse the effects of TF5 and the apoptosis inducers

etoposide (VP-16) and sphingomyelinase. That is, the clonogenic potential of the cells was not protected in any of the test groups. Since it has been recently reported that (1) zVAD-fmk fails to protect HL-60 cells from other apoptosis inducers (Zhang, Sheng and Loh, 1999) and (2) zVAD-fmk did not rescue the clonogenic potential of HL-60 cells from apoptosis induced by etoposide, staurosporine and Actinomycin-D (Amarente-Mendes et al., 1998), these experiments were repeated using another known apoptosis inducer that acts through a distinct mechanism, sphingomyelinase (Smase). Again neither the effects of TF5 nor of Smase were reversed by zVAD-fmk. Therefore, cytological analysis by light microscopy and the TUNEL assay were used to further investigate the mechanism of TF5 action on HL-60 cells. These experiments confirm that apoptosis is not involved. Thus, TF5 appears to induce cytostasis in HL-60 cells. This is in contrast to our observations with the MMQ and C6 neuroendocrine cell lines, in which apoptosis was the apparent mechanism of TF5 inhibition. The data in our study may demonstrate a new biological property of TF5: the inhibition of cell proliferation and the induction of cytostasis in human myeloblastic leukemia cells *in vitro*.

Identification of TF5 Anti-Proliferative Activity

We believe that the anti-proliferative activity in TF5 is due to one or more peptide components. On the basis of dry weight, TF5 is 99% protein with a small amount of carbohydrate and essentially no nucleotide (Hooper et al., 1975). Several previously purified components of TF5 (*i.e.* $T\alpha_1$, $T\beta_4$, MB-35) had no discernible effect on HL-60 cell proliferation. Therefore, TF5 was further fractionated using rpHPLC. Further purification of TF5 into sixty one-minute fractions by rpHPLC resulted in one fraction

(fraction 24) that significantly inhibited HL-60 cell proliferation. It is noteworthy that none of the previously purified thymosin peptides elute in this fraction. Fraction 24 was ten times more potent than TF5, indicating substantial purification of the anti-proliferative activity. The rpHPLC process did not provide enough material for further purification, but it does support our argument that the observed inhibition of HL-60 cells is due to an activity present in TF5 rather than a non-specific effect (i.e. not all fractions affected the cells).

To generate useful quantities of the anti-proliferative activity in TF5, it was fractionated by size exclusion gel permeation HPLC. None of the resulting fractions consistently demonstrated significant inhibition of HL-60 cell proliferation. This may indicate that a component necessary for biological activity was removed, or that the fractionation process modified the active molecule.

To avert this possibility and to generate larger sample quantities, we separated substantial quantities of biologically active TF5 fractions on the basis of molecular size by gel filtration column chromatography on a Sephadex gel bed. Although the fabrication process for TF5 extract excludes proteins larger than 15 kDa, we separated biologically active fractions of TF5 corresponding to approximately 20 kDa that inhibited HL-60 cell proliferation by more than 80%. This may suggest that oligomer formation occurs in a biologically active form of thymosin components. We also obtained moderately active (*i.e.* fraction 34, Fig 20) fractions of approximately 1200- 1500 MW. Data obtained by Hooper and colleagues (1975) also suggested the presence of subunits in purified fractions of TF5. They purified a moderately active molecule with a calculated molecular weight of 12,200 from TF5. When this preparation was subjected to gel filtration under

reducing and denaturing conditions, it eluted as two protein peaks of MW 3200 and 2400, suggesting that the preparation is composed of polypeptide subunits.

Estimates of molecular weight were performed under reducing and denaturing conditions on polyacrylamide gels. Separation on 4- 20% Tris-Glycine polyacrylamide gels did not substantially resolve the fractions, which migrated as a dense group of bands in the range of 6 kDa to 22 kDa. This suggests that the fractions may contain multiple peptides of similar molecular weight. Pre-cast 12% Bis-Tris polyacrylamide gels were used to achieve better separation of very low molecular weight components. On these gels, TF5 and active fractions of TF5 show a strong band density at approximately 6 kDa, and three other predominant bands in the 5000-15000 molecular weight range.

To determine whether the anti-tumor activity present in TF5 can be isolated from other thymus extracts, we compared the anti-proliferative effects of TF5 on HL-60 cells to the effects exerted by less purified bovine thymus preparations. Thymus acetone preparations (acetone precipitation) inhibited HL-60 cell proliferation in a dose-dependent manner. One such preparation, TAP, was subjected to reverse-phase HPLC. The activity profile for these fractions has two regions of inhibition, and a large peak of stimulation. The area of greatest anti-proliferative activity, TAP fraction 20, corresponds to the protein peak, while the growth-stimulating activity (fraction 10) precedes it. We hypothesized that separation of the two activities would allow more pronounced concentration-dependent effects of the anti-proliferative action of TAP fraction 20 (TAP-20) on HL-60 cells. TAP-20 was 40 times more effective than TAP in the inhibition of HL-60 cell proliferation. As with TF5, we were unable to purify this activity further by size-exclusion HPLC. Given the substantially concentrated anti-proliferative activity in

this preparation, alternative methods of purification (*i.e.* gel filtration column chromatography) seem warranted. Likewise, two-dimensional isoelectric focusing on SDS-PAGE gels would provide better resolution of the biologically active TF5 fractions.

Conclusions

We conclude that TF5 inhibits the proliferation of HL-60 human myelogenous leukemia cells in a dose and time-dependent manner *in vitro*. This inhibition does not seem to be due to a nonspecific effect. Nor does it appear that a factor secreted by the cells disrupts the effects of TF5. Our observations suggest the HL-60 cells metabolize a consumable factor in TF5. Thymosin also inhibited the colony-forming potential of HL-60 cells in soft agar. We suggest that a thymic peptide may prevent metastatic growth of human leukemia cells *in situ*. TF5 apparently does not induce apoptosis in the HL-60 cells, but it may induce cytostasis. Thus, our observations may demonstrate a new biological property of TF5: the inhibition of cell proliferation and colony-forming potential, and the induction of cytostasis in HL-60 human myelogenous leukemia cells *in vitro*.

Useful quantities of a low molecular weight, biologically active TF5 activity can be separated by gel filtration chromatography. Further purification is necessary to identify and sequence the active component(s). None of the previously purified thymosin peptides elutes in the biologically active rpHPLC fraction of TF5. However, TF5-like activity was observed in several thymus acetone powders that are less purified than TF5.

These studies suggest that a thymic hormone immune surveillance mechanism may suppress the onset of certain types of leukemia. The isolation of the active thymosin peptide may provide a novel treatment strategy for the management of certain leukemias.

APPENDIX I

TABLES

Effect of TF5 on HL-60 Cell Viability

Group	Time (hr)		
	24	48	72
Vehicle	96.6 ± 0.5	96.9 ± 0.7	97.5 ± 0.4
100 μg/mL	93.8 ± 0.9	96.9 ± 0.7	96.4 ± 0.6
400 μg/mL	91.5 ± 1.8	91.2 ± 0.5	92.5 ± 0.9
800 μg/mL	76.4 ± 3.0	61.4 ± 5.2	64.2 ± 5.3

Table 1. Effect of TF5 on the viability of HL-60 human myelogenous leukemia cells. 2.4 x10^5 HL-60 cells were exposed to TF5 for 72 hr. Results are the averages of percent viability from three independent experiments.

Treatment	DNA Damage (FM)	Cell Death (LM)
	% Vehicle Control	
Vehicle	1 +/- 1%	2 +/- 1%
Ceramide	5 +/- 2%	27 +/- 1%
Sphingosine	11 +/- 4%	89 +/- 1%

Table 2. Cytological Effects of Sphingolipid Messengers on HL-60 Cells. Cells were exposed to vehicle or ceramide or sphingosine (10 μM) for 9 hr. DNA damage was visualized by fluorescence microscopy (FM) after staining with FITC-dUTP in the presence of TdT. Morphologic features of apoptotic cell death were observed by light microscopy (LM) after staining with conventional Wright-Giemsa. Values are expressed as the mean ± SEM of four determinations.

Treatment	DNA Damage (FM)	Cell Death (LM)
	% Vehicle Control	
CONTROL	3 +/- 1%	2 +/- 1%
TF5 24 Hrs.	3 +/- 1%	2 +/- 1%
TF5 48 Hrs.	5 +/- 2%	1 +/- 1%
TF5 72 Hrs.	4 +/- 1%	4 +/- 2%

Table 3. Cytological Effects of TF5 on HL-60 Cells. Cells were exposed to a maximal dose of TF5 for 72 hr. Cells exhibiting DNA damage were visualized by fluorescence microscopy (FM) after staining with FITC-dUTP in the presence of TdT. Morphologic features of apoptotic cell death were observed by light microscopy (LM) after staining with conventional Wright-Giemsa. Values are expressed as the mean ± SEM of four determinations.

APPENDIX II

FIGURES

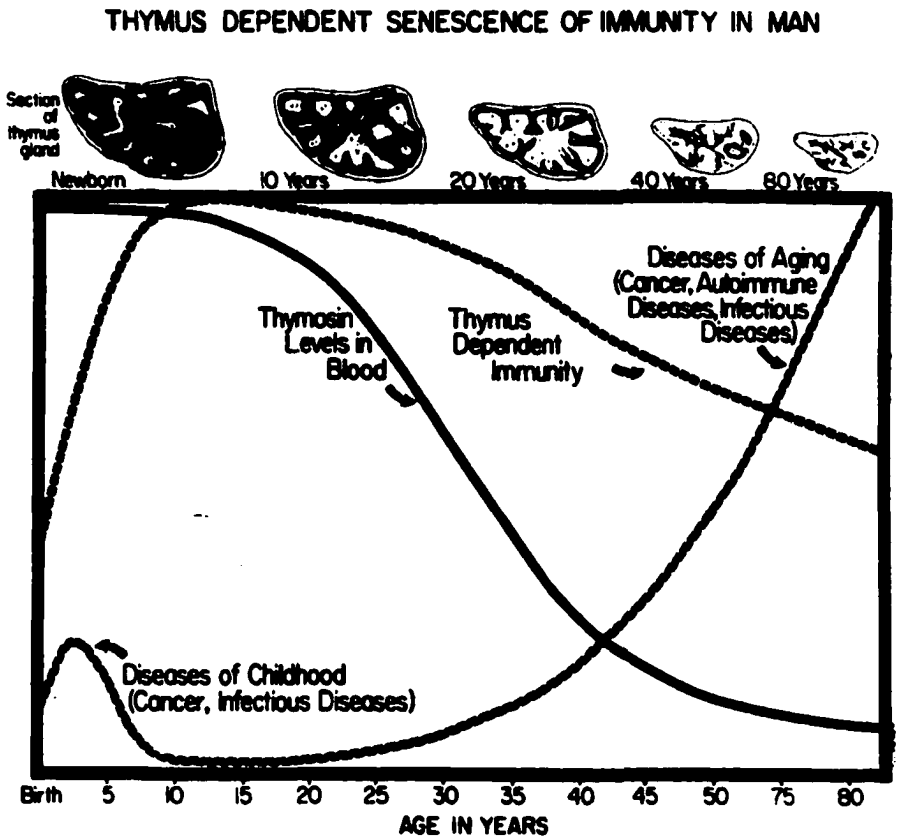

Figure 1. Thymus Dependent Senescence of Immunity in Man. **Schematic showing the relationship between decreased thymus function, thymic hormone levels, and thymus-dependent immunity.**

***Reprinted from Goldstein et al., 1975, by permission of the author.**

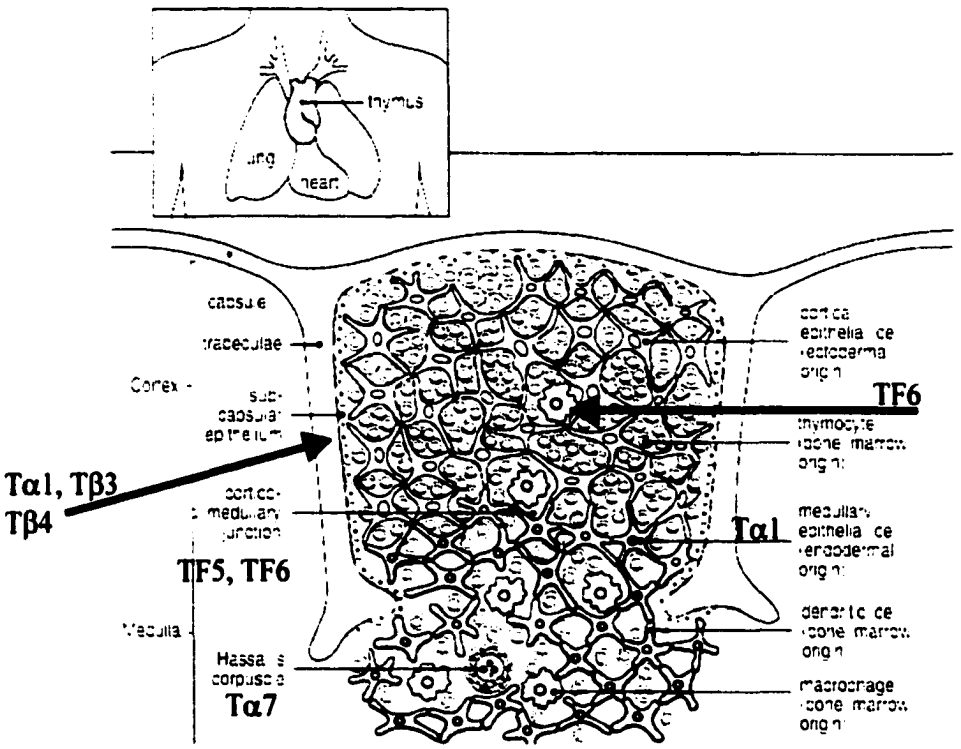

Figure 2. Schematic showing the locations of thymosin peptides in the thymus Gland. TF5 and TF6 have been found throughout the cortex and medullary epithelial cells (Hirokawa, McClure, and Goldstein, 1982; Schuurman et al, 1985).

Adapted from Janeway and Travers, 1994

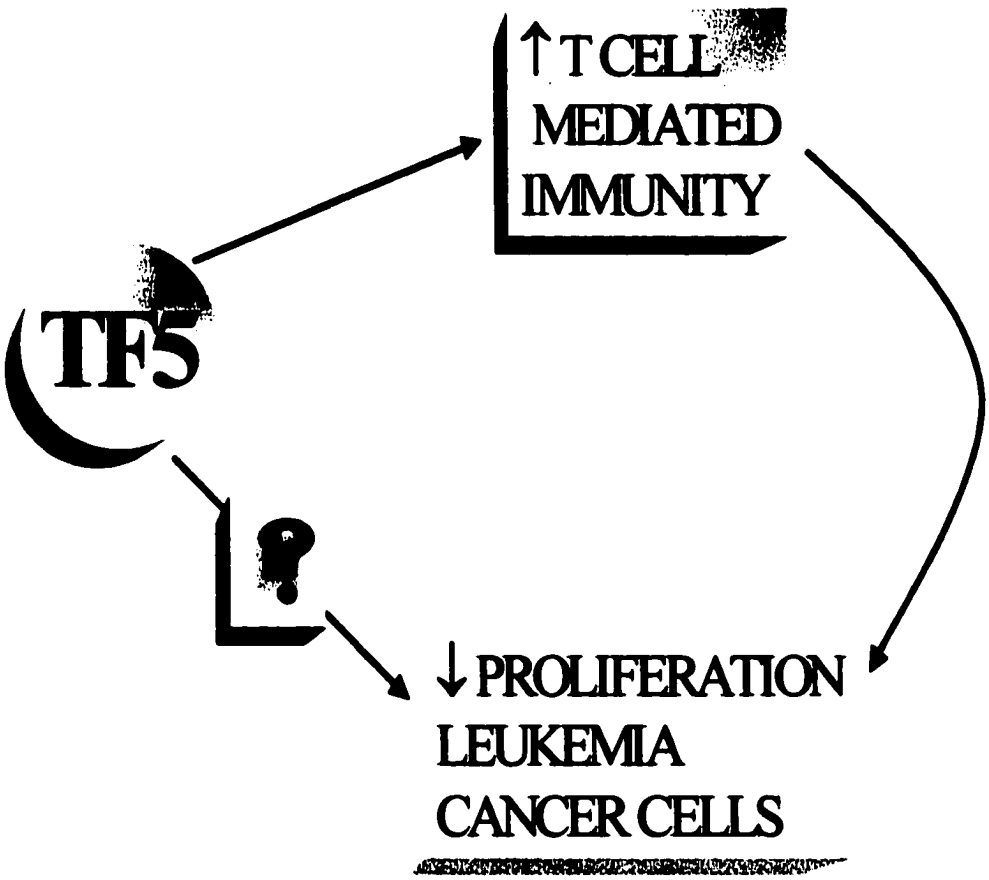

Figure 3. Principle hypothesis of this study. Because TF5 generally stimulates thymus-dependent immunity, and because ant-tumor immunity is under thymic control, we hypothesized that TF5 would directly inhibit leukemia cell proliferation.

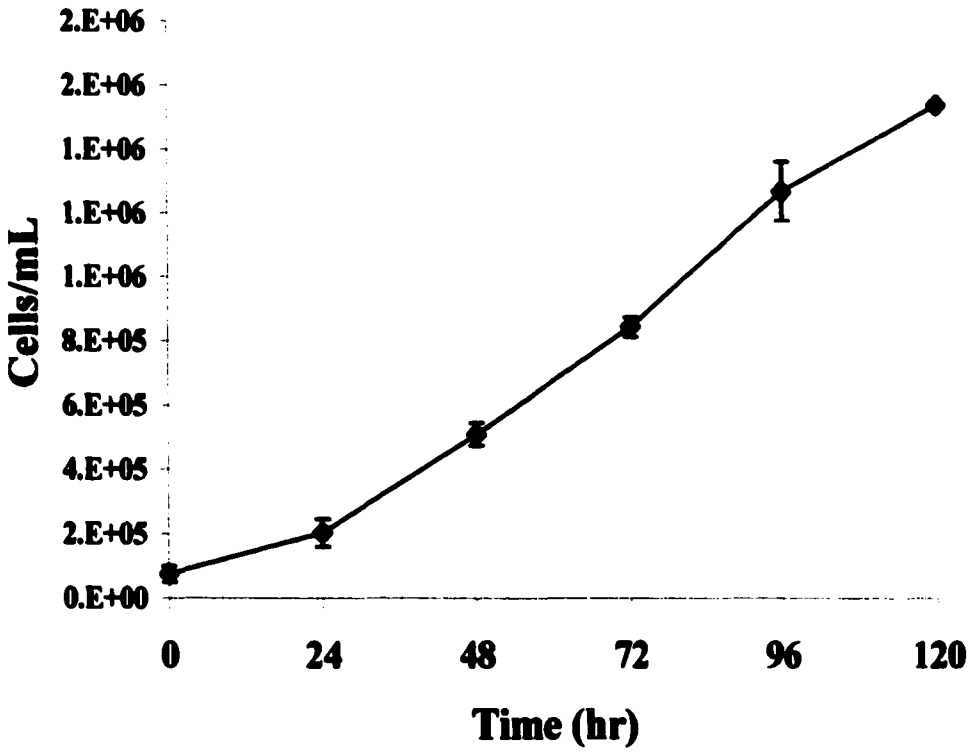

Figure 4. Proliferation of HL-60 human myelogenous leukemia cells in continuous suspension culture. HL-60 cells (75 x 10^3 cells/well) were cultured in a humidified atmosphere of 5% CO_2-95% air at 37°C in phenol-free RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum, sodium pyruvate, nonessential amino acids and antibiotics. Cell numbers were determined by standard microscopy every 24 hr. The mean doubling time for the data shown is 28.5 hr. The data are expressed as the mean ± SEM of groups consisting of four observations.

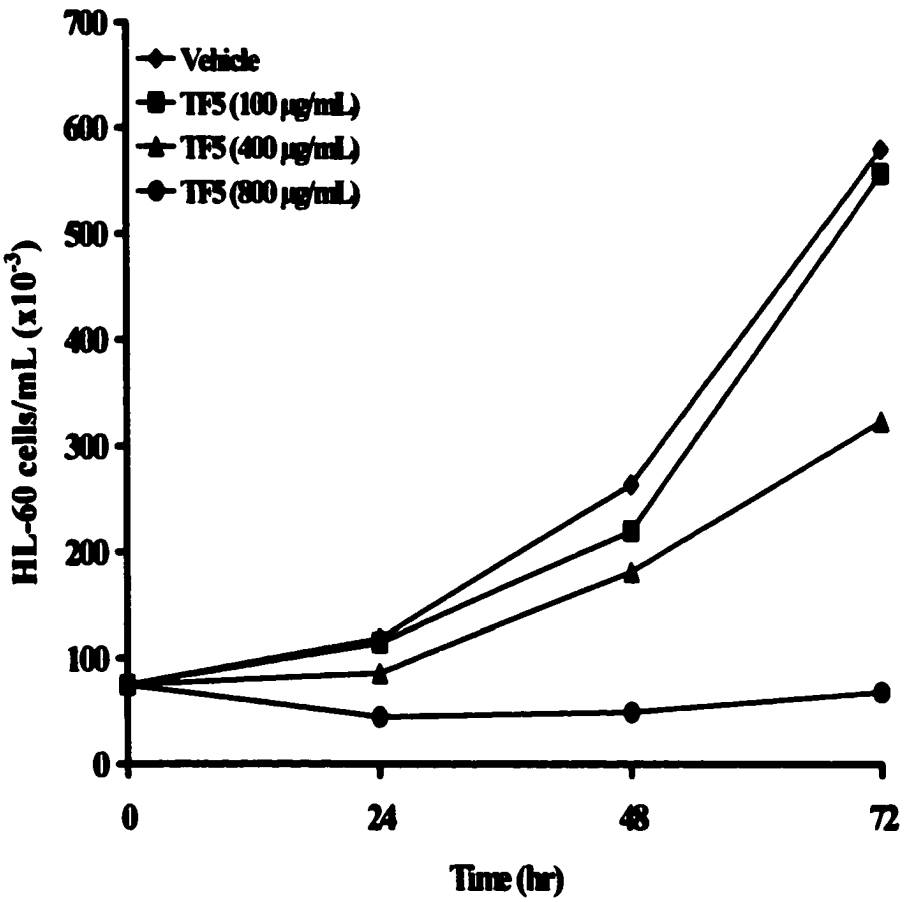

Figure 5. TF5 inhibits HL-60 human myelogenous leukemia cell proliferation. Cultured HL-60 cells (75 x 10^3 cells/well) were exposed to either vehicle (complete RPMI-1640) or different concentrations of TF5 for 24-72 hr, and cell concentrations were determined by standard microscopy and viable dye exclusion.

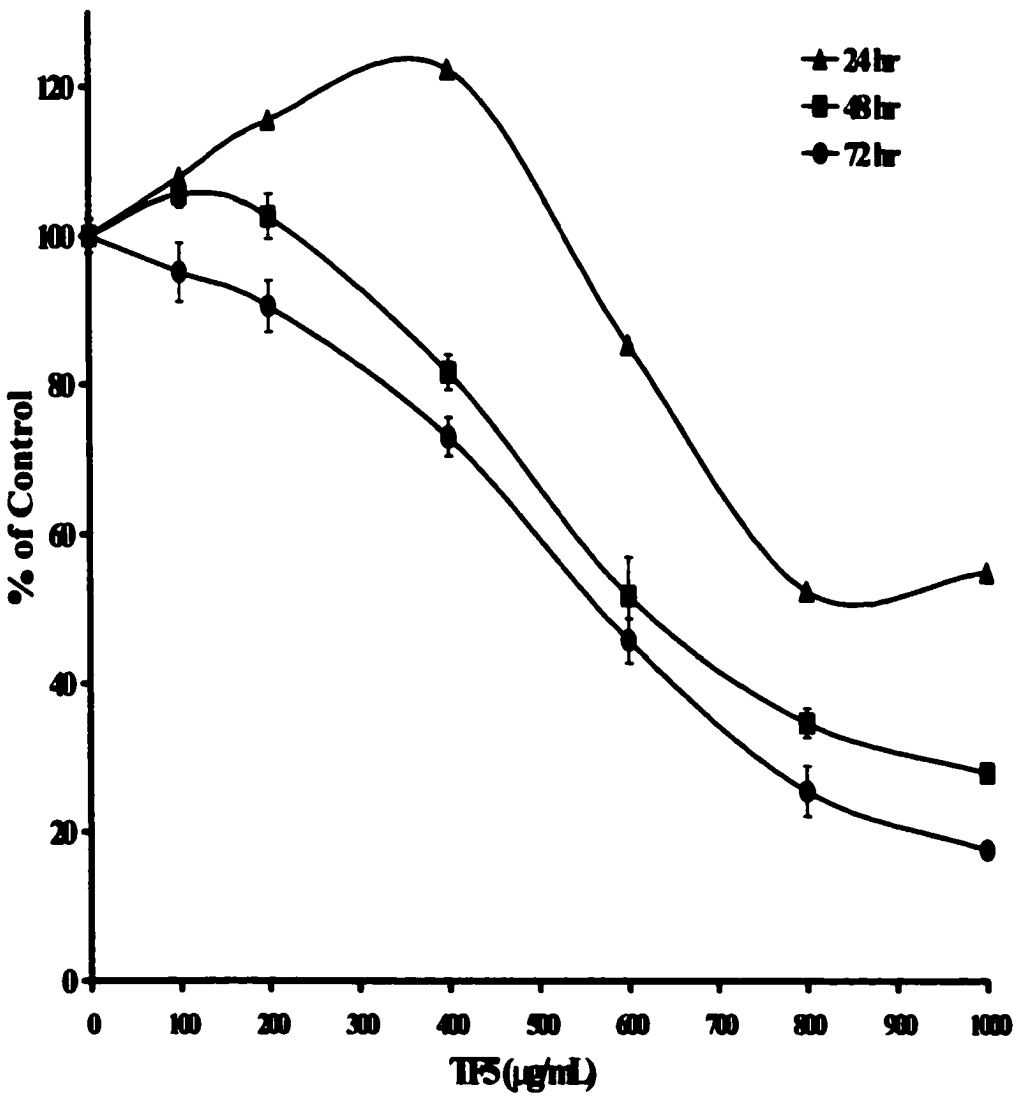

Figure 6. TF5 inhibits HL-60 human myelogenous leukemia cell proliferation: effect of incubation period. Cultured HL-60 cells (20 x 10^3 cells/well) were exposed to either vehicle (complete RPMI-1640) or different concentrations of TF5 for 24-72 hr, and cell proliferation was determined by the MTT viability assay.

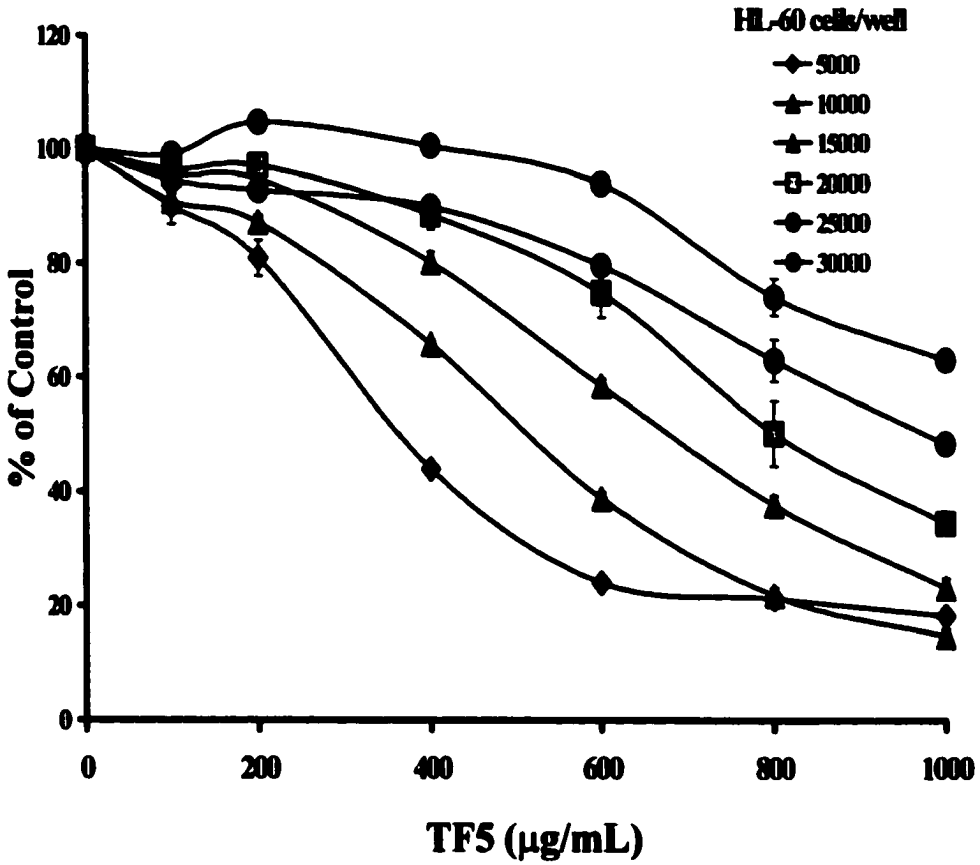

Figure 7. TF5 inhibits HL-60 human myelogenous leukemia cell proliferation: effect of cell number. Cultured HL-60 cells (5-30 x 10^3 cells/well) were exposed to either vehicle (complete RPMI-1640) or different concentrations of TF5 for 72 hr, and cell proliferation was determined by the MTT viability assay.

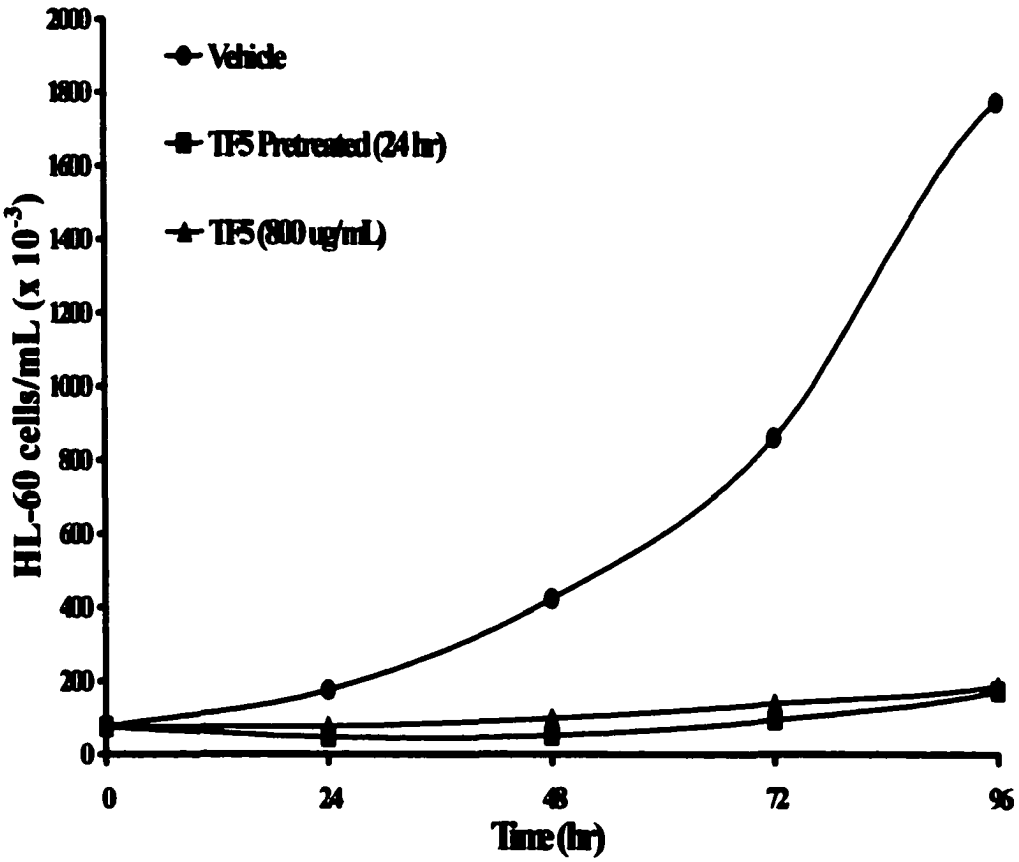

Figure 8. TF5 inhibits HL-60 human myelogenous leukemia cell proliferation: effect of pretreatment period. Cultured HL-60 cells (80 x 10^3 cells/mL) were pretreated with 800 μg/mL TF5 for 24 hr and cultured in complete RPMI-1640, or cells were exposed to either vehicle or 800 μg/mL TF5. Cell concentrations were determined by standard microscopy and viable dye exclusion at 24 to 96 hr.

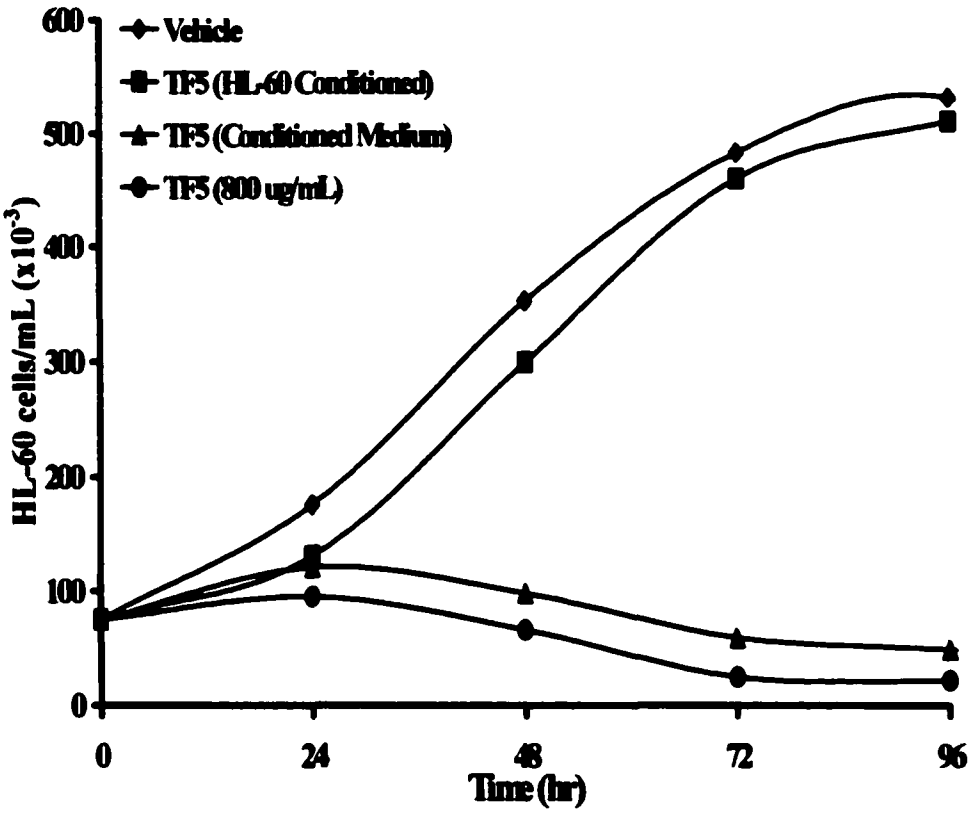

Figure 9. TF5 inhibits HL-60 human myelogenous leukemia cell proliferation: effect of HL-60 conditioning of TF5. Cultured HL-60 cells (80 x 10^3 cells/well) were either treated with vehicle (complete RPMI-1640), TF5 (800 μg/mL), or TF5 that was removed from HL-60 cells following a 24 hr incubation period. In addition, HL-60 cells were exposed to TF5 that was diluted in a 24 hr HL-60 cell- conditioned medium. Cell concentrations were determined by standard microscopy and viable dye exclusion.

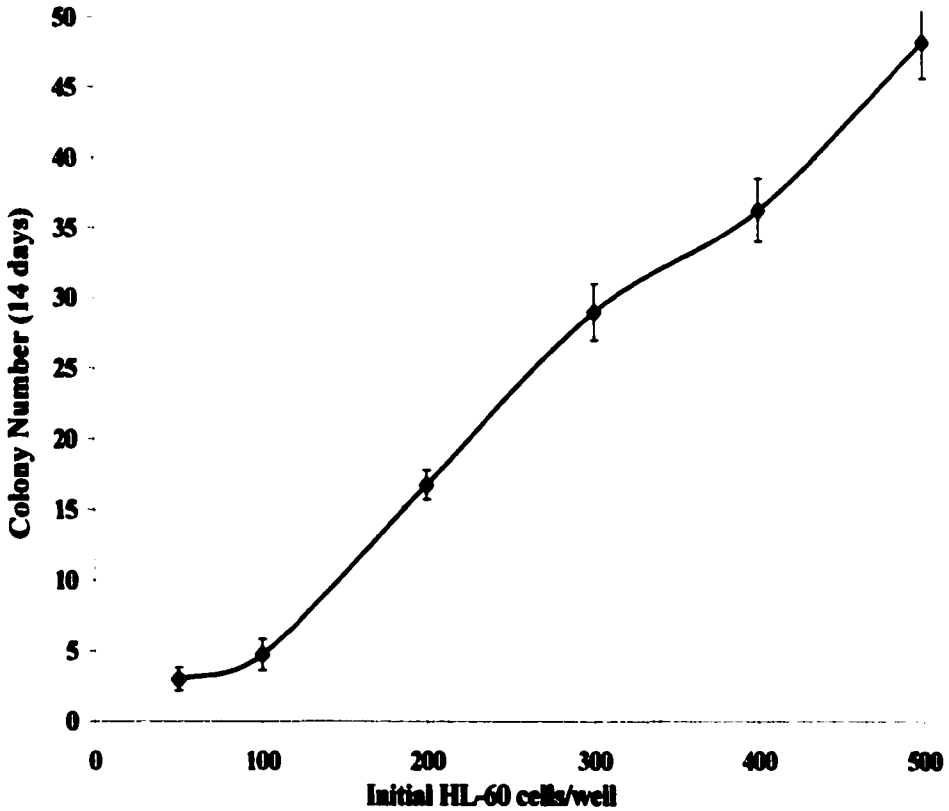

Figure 10. HL-60 human myelogenous leukemia cell colony-forming potential in soft agar is dependent on initial cell plating density. Cultured HL-60 cells were plated at varying initial cell numbers and then cultured for 14 days in agar.

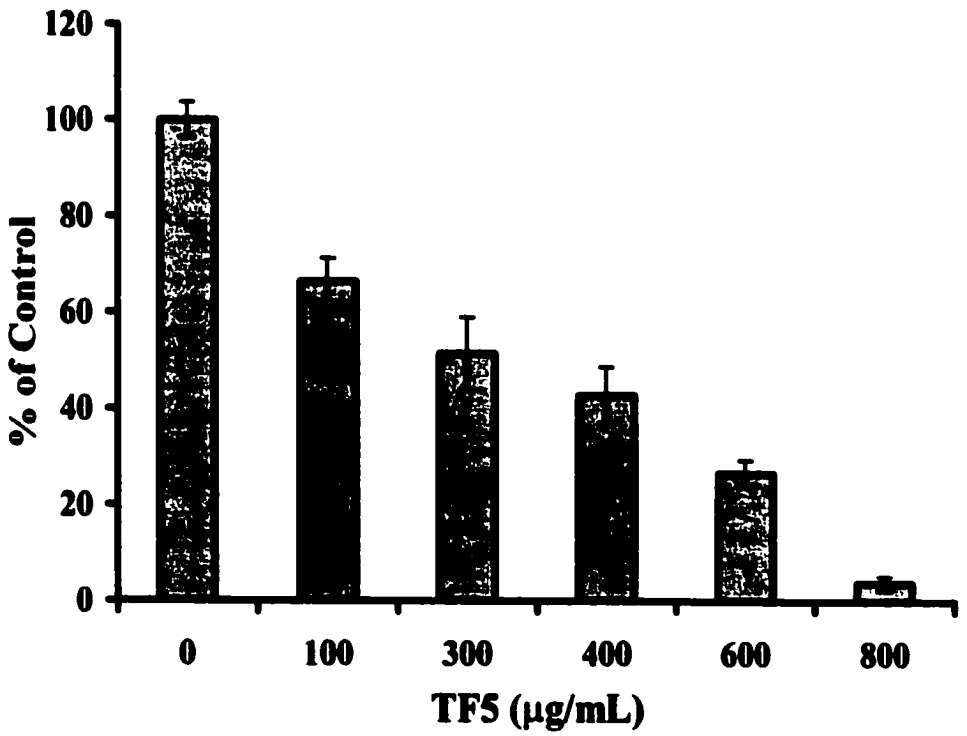

Figure 11. TF5 inhibits the clonogenic potential of HL-60 human myelogenous leukemia cells: effect of TF5 dose. Cultured HL-60 cells (100 x 10^3 cells/mL) were pretreated with 100-800 μg/mL TF5 for 24 hr and then cultured for 14 days in agar (300 cells/well).

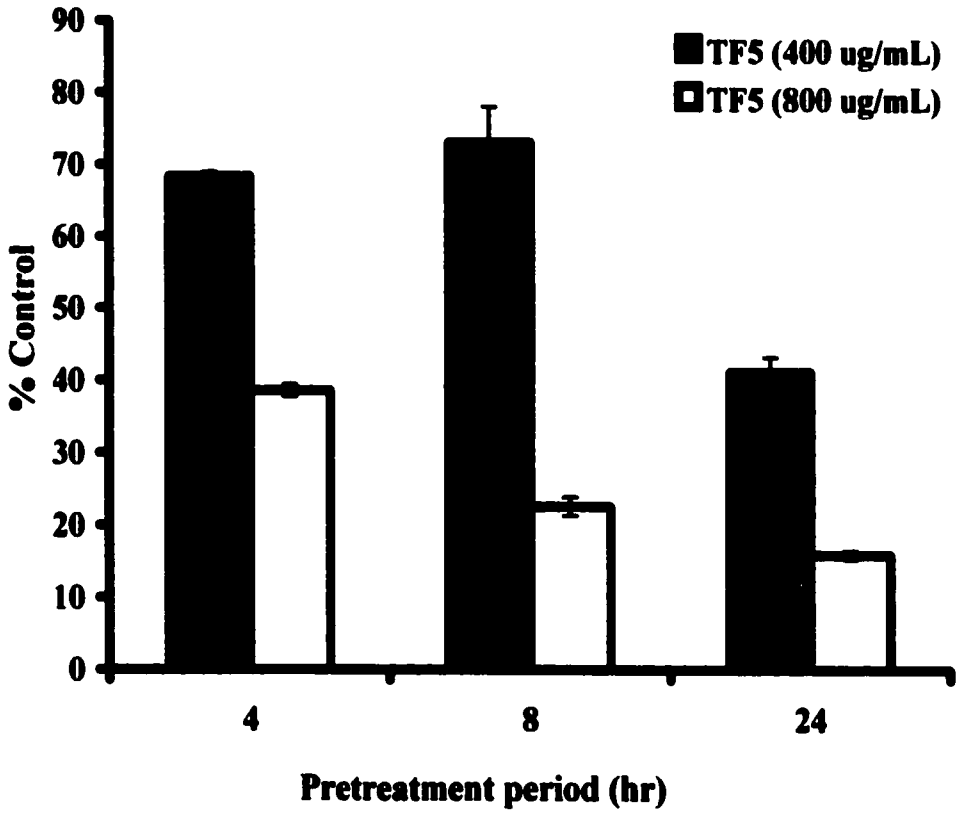

Figure 12. TF5 inhibits the clonogenic potential of HL-60 human myelogenous leukemia cells: effect of pretreatment period. Cultured HL-60 cells (100×10^3 cells/mL) were pretreated with 800 μg/mL TF5 for 4, 8 or 24 hr and then cultured for 14 days in agar.

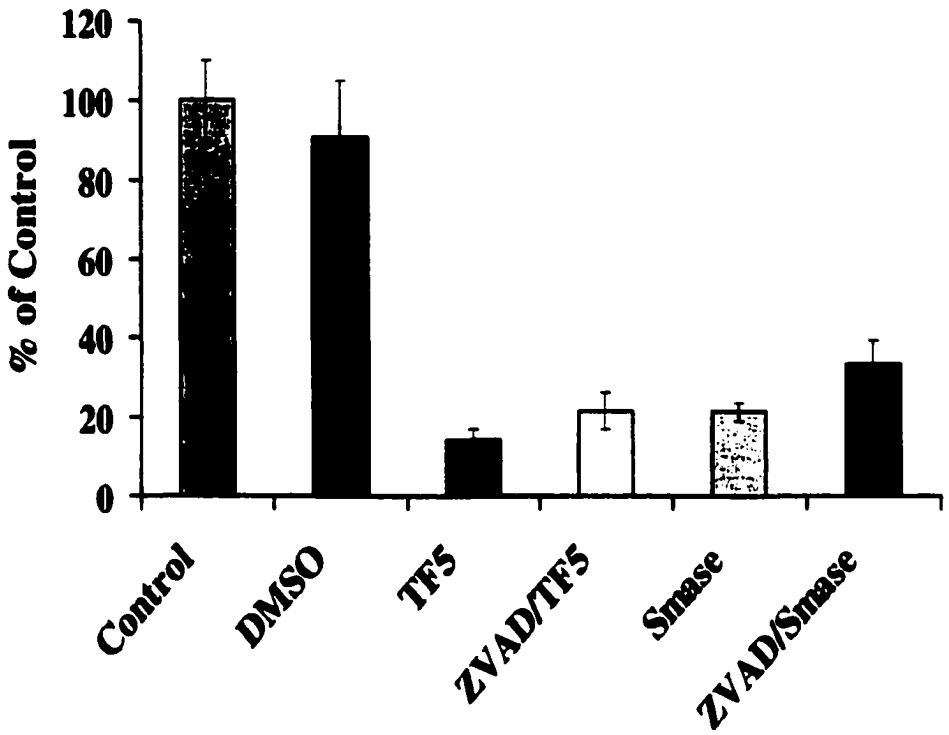

Figure 13. TF5 inhibits the clonogenic potential of HL-60 human myelogenous leukemia cells: effect of pre-incubation of HL-60 cells with an apoptosis inhibitor. Cultured HL-60 cells (100 x 10^3 cells/mL) were pretreated with 800μg/mL TF5 for 24 hr or pre-incubated in the presence of 100μM zVAD-fmk (in DMSO) and then pretreated with 800μg/mL TF5 for 24 hr or 200mU sphingomyelinase for 9 hr. The cells were then cultured for 14 days in agar. Colony numbers were determined by microscopy.

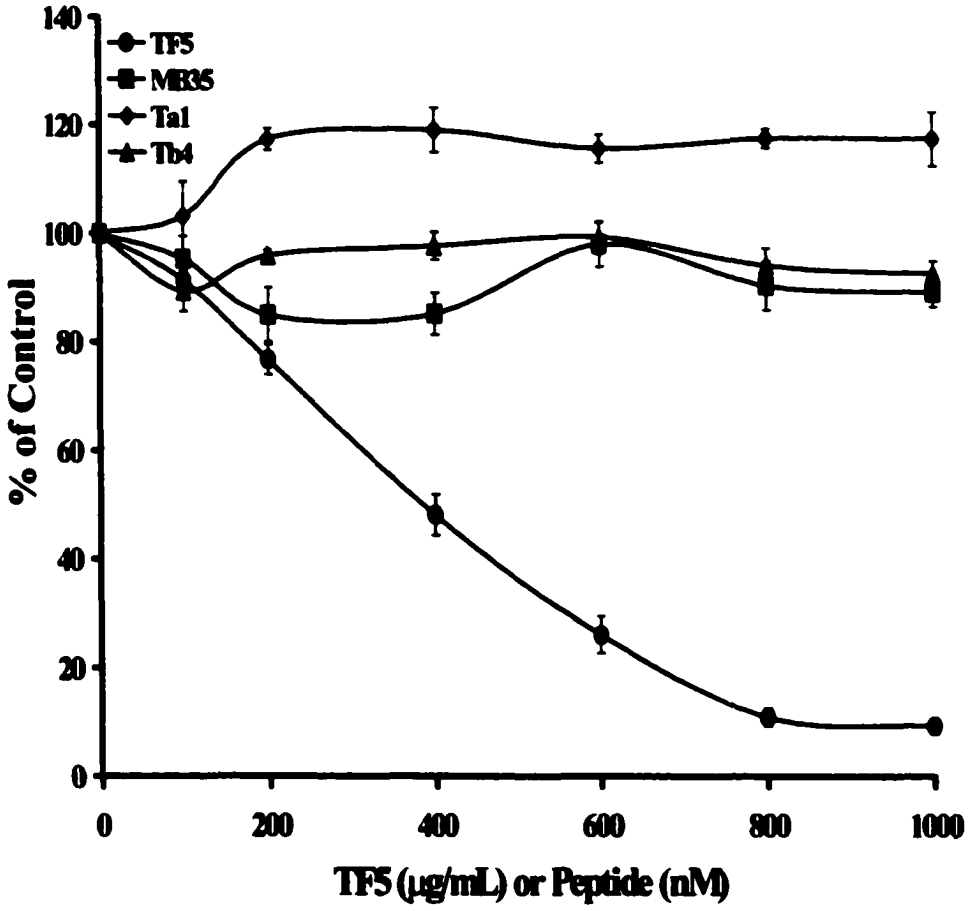

Figure 14. Effect of purified thymosin peptides on HL-60 human myelogenous leukemia cell proliferation: comparison to TF5. Cultured HL-60 cells (20 x 10^3 cells/well) were exposed to vehicle (RPMI-1640, supplemented) or TF5, $T\alpha_1$, $T\beta_4$ or MB-35 for 72 hr. Only TF5 significantly suppressed cell proliferation. The data are expressed as the mean ± SEM of groups consisting of four observations.

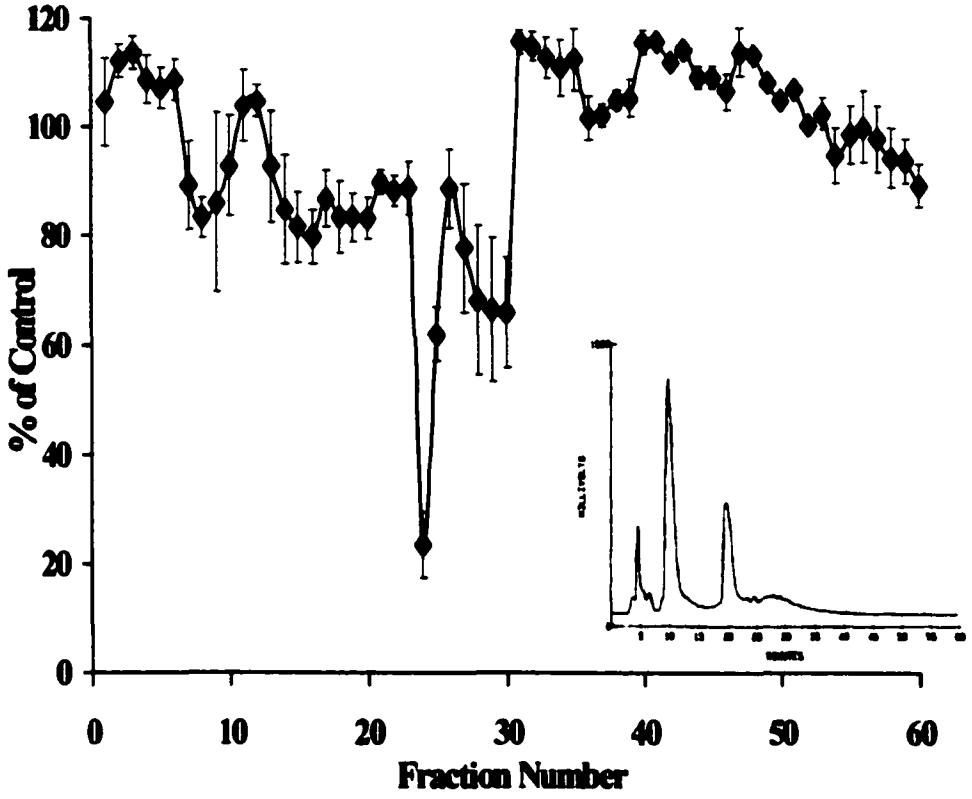

Figure 15. Effect of HPLC fraction 24 on HL-60 human myelogenous leukemia cell proliferation: comparison to TF5. Cultured HL-60 cells (20 x 10^3 cells/well) were exposed to vehicle (complete RPMI-1640), fraction 24 or TF5 for 72 hr, and cell proliferation was determined by the MTT viability assay. Inset shows the UV profile at 280nm of the HPLC separation of TF5.

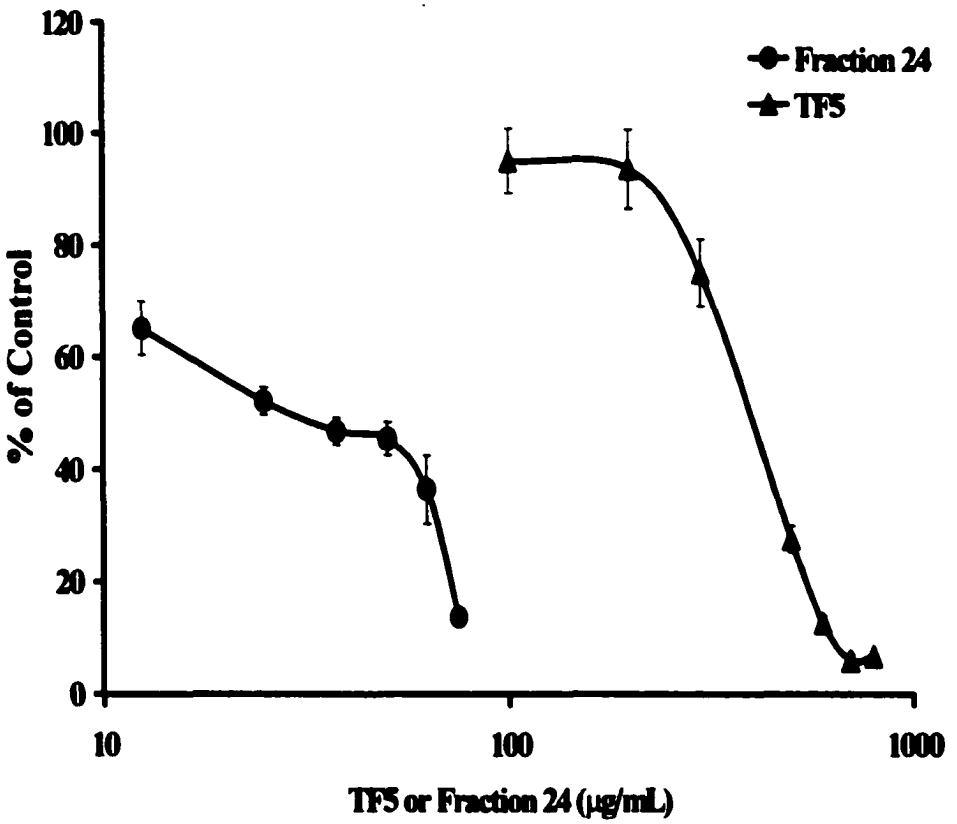

Figure 16. Effect of HPLC fraction 24 on HL-60 human myelogenous leukemia cell proliferation: comparison to TF5. Cultured HL-60 cells (20 x 10^3 cells/well) were exposed to vehicle (complete RPMI-1640), fraction 24 or TF5 for 72 hr, and cell proliferation was determined by the MTT viability assay.

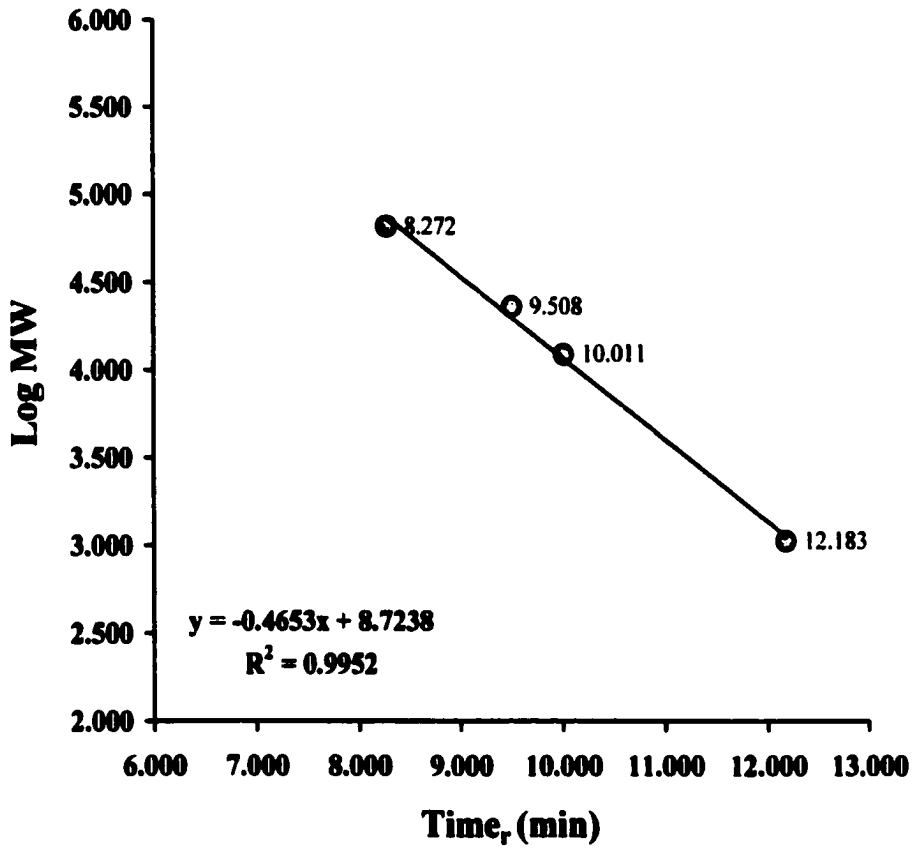

Figure 17. Protein standard curve for size exclusion HPLC. Retention times are plotted against the log of the MW of each protein standard (BSA, trypsin, cytochrome c, and bradykinin). The data are expressed as the mean ± SEM of groups consisting of three separate sample injections.

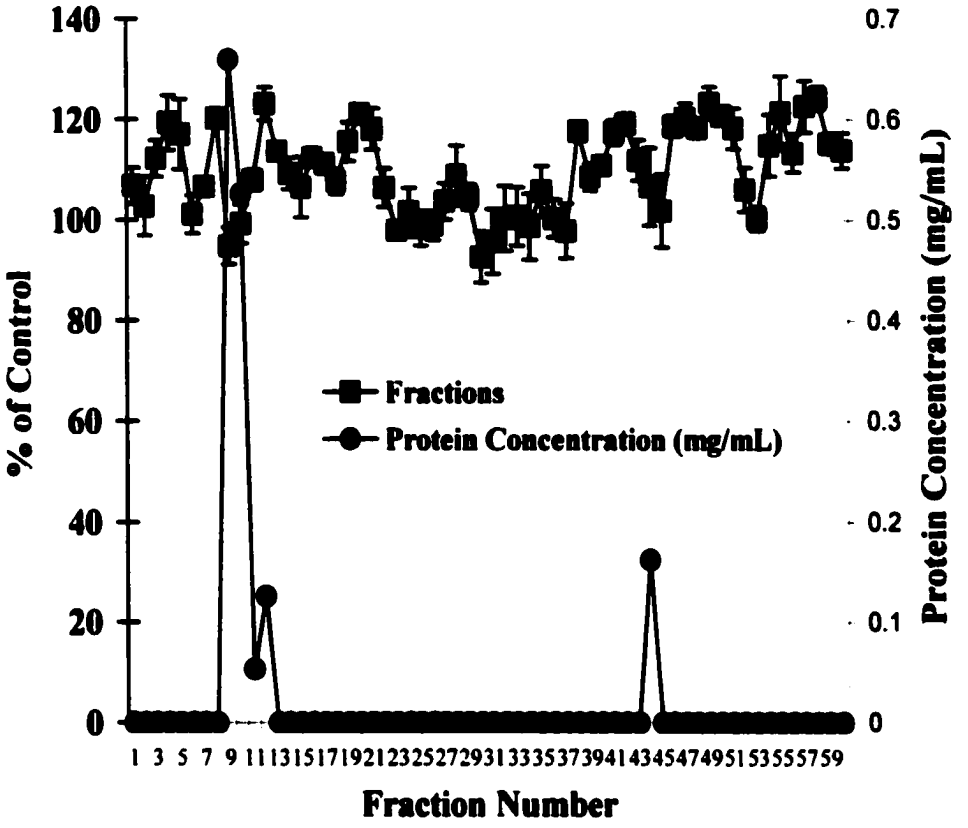

Figure 18. Effect of HPLC fractionated TF5 on HL-60 human myelogenous leukemia cell proliferation. Cultured HL-60 cells (20 x 10^3 cells/well) were exposed to either vehicle (complete RPMI-1640) or different fractions of size exclusion HPLC-separated TF5 for 72 hr, and cell proliferation was determined by the MTT viability assay.

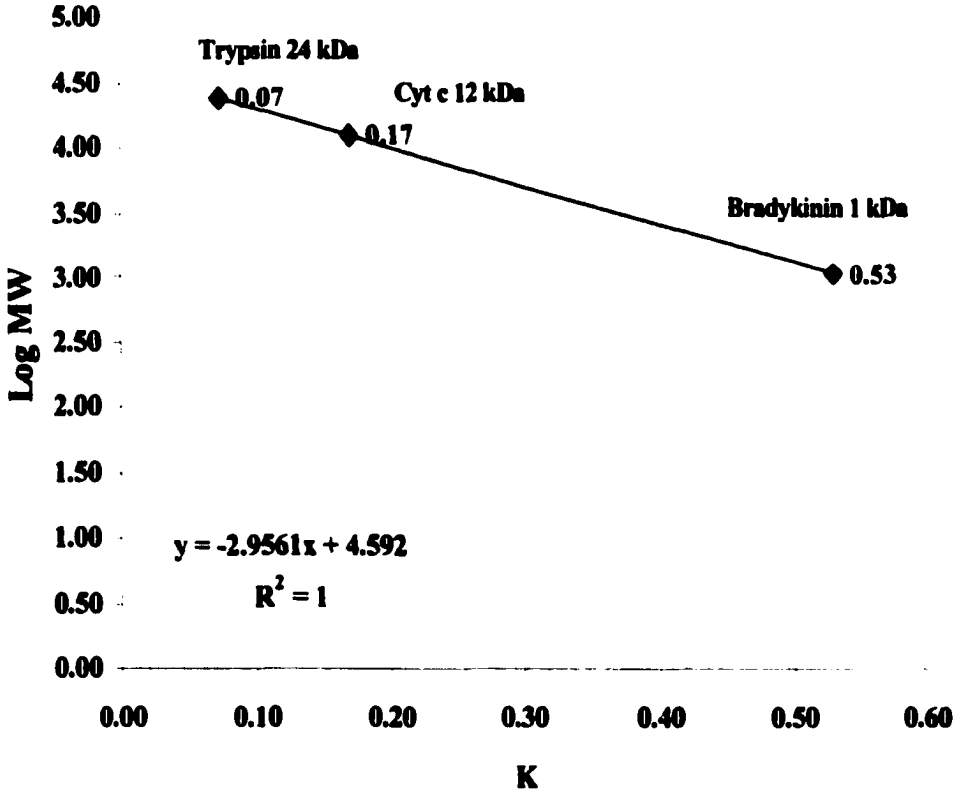

Figure 19. Protein standard curve for gel filtration chromatography. Distribution coefficient (K) values for the protein standards trypsin (MW 24 kDa), cytochrome c (MW 12kDa), and bradykinin (MW 1060 Da) are on Sephadex G-50 are shown. The flow rate was 1.40 mL/min.

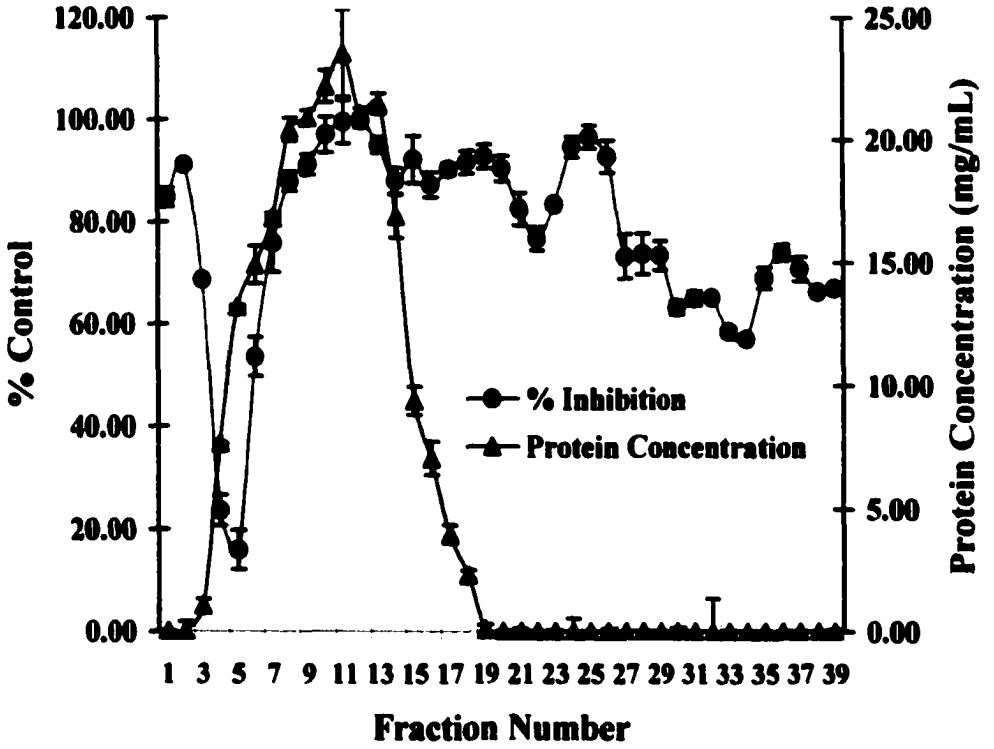

Figure 20. Effect of gel filtration chromatography- separated TF5 fractions on HL-60 human myelogenous leukemia cell proliferation: comparison to Lowry protein values. TF5 was loaded onto the column (50 mg) and 1-minute fractions were collected. Protein concentrations were determined by the Lowry method. Cultured HL-60 cells (20 x 10^3 cells/well) were exposed to individual fractions for 72 hr and cell proliferation was determined by the MTT reaction assay. The cell proliferation data are expressed as the mean ± SEM of groups consisting of four observations.

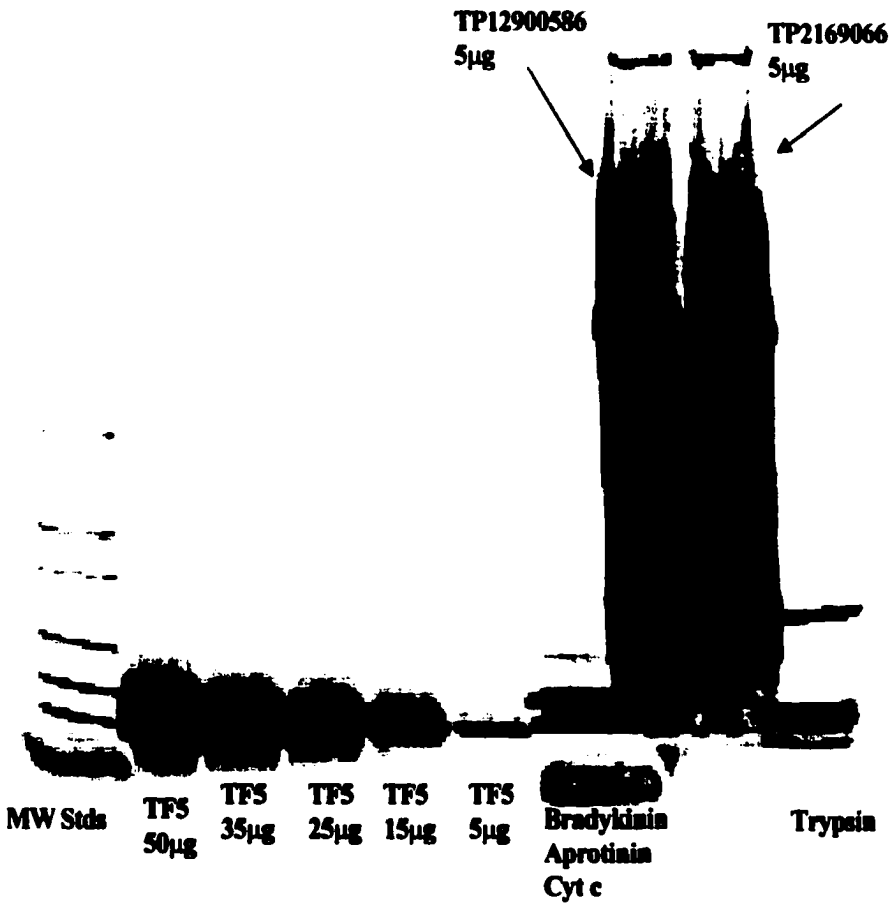

Figure 21. Separation of TF5 and thymus preparations (TP) on 4-20% Tris-glycine gels. TF5 (5-50 μg) and thymus preparations (5 μg) were reduced with β-mercaptoethanol and electrophoresis was carried out at 125V (constant) for approximately 60 minutes. Molecular weight standards (MW Stds) are 200 kDa to 2.5 kDa.

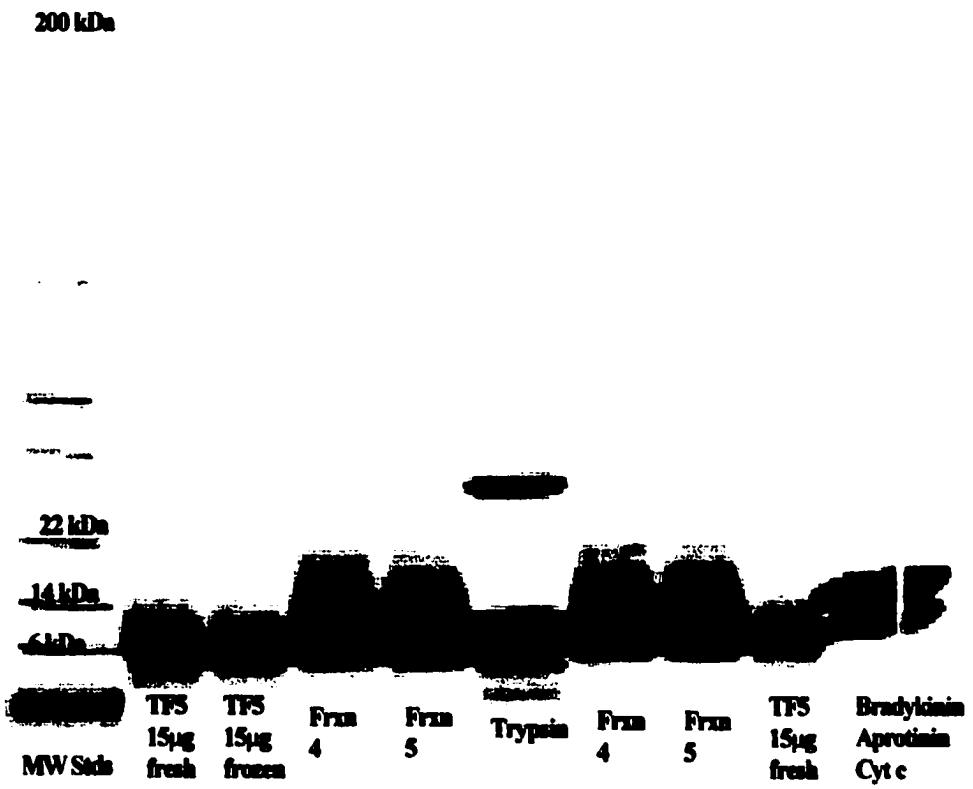

Figure 22. Separation of TF5 and active TF5 gel filtration chromatography fractions on 4-20% SDS- PAGE gels. Samples were reduced with β-mercaptoethanol and electrophoresis was carried out at 125V (constant) for approximately 60 minutes.

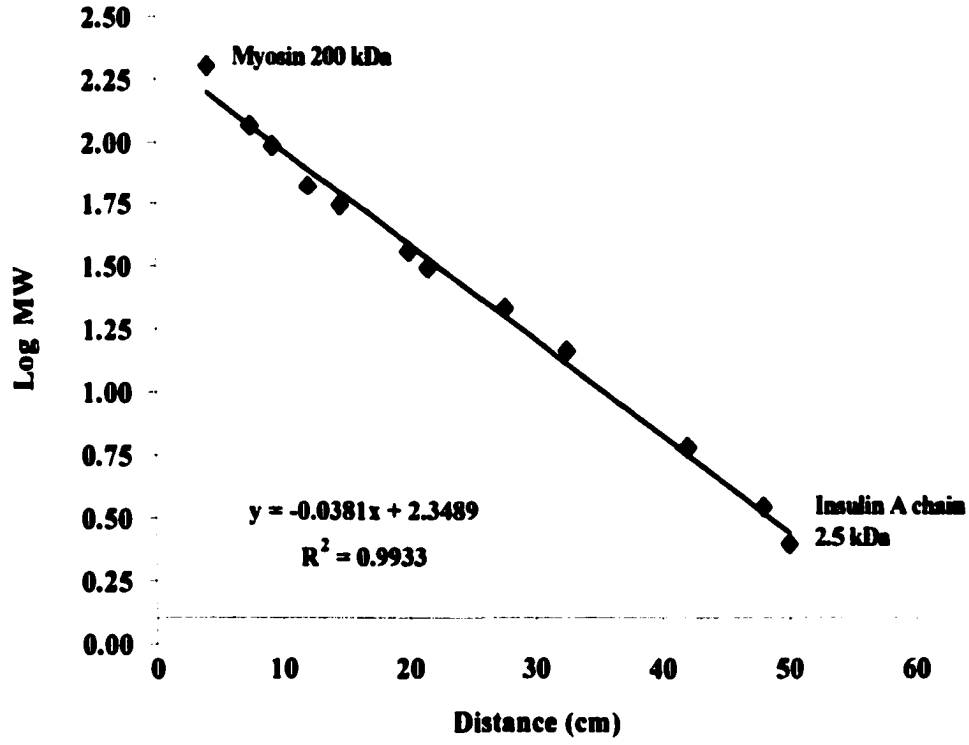

Figure 23. Protein standard curve for gel electrophoresis on 12% Bis-tris gels. Electrophoresis was carried out at 200V (constant) for approximately 30 minutes.

Figure 24. Separation of TF5 and active TF5 gel filtration chromatography fractions on 12 %Bis-Tris polyacrylamide gels. 3-5µg of sample was loaded onto each well, and electrophoresis was carried out at 200V (constant) for approximately 30 minutes. Fractions 2 and 3 are from the separation of 10mg of TF5. Fractions 4, 5, and 6 are from the separation of 50 mg of TF5.

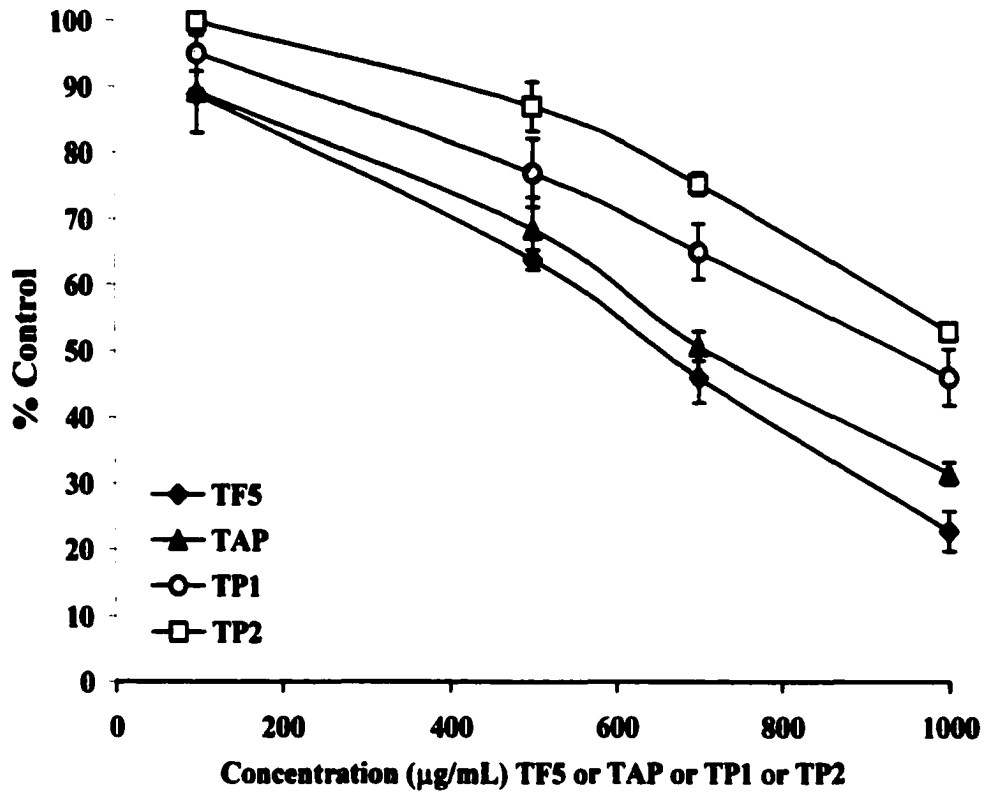

Figure 25. Effects of TF5 and thymus acetone preparations on HL-60 human myelogenous leukemia cell proliferation. Cultured HL-60 cells (20 x 10^3 cells/well) were exposed to vehicle (RPMI-1640, supplemented) or TF5, TAP, TP1 or TP-2 for 72 hr and then proliferation was measured by the MTT reaction assay. All thymus preparations inhibited HL-60 cell proliferation. The data are expressed as the mean ± SEM of groups consisting of four observations.

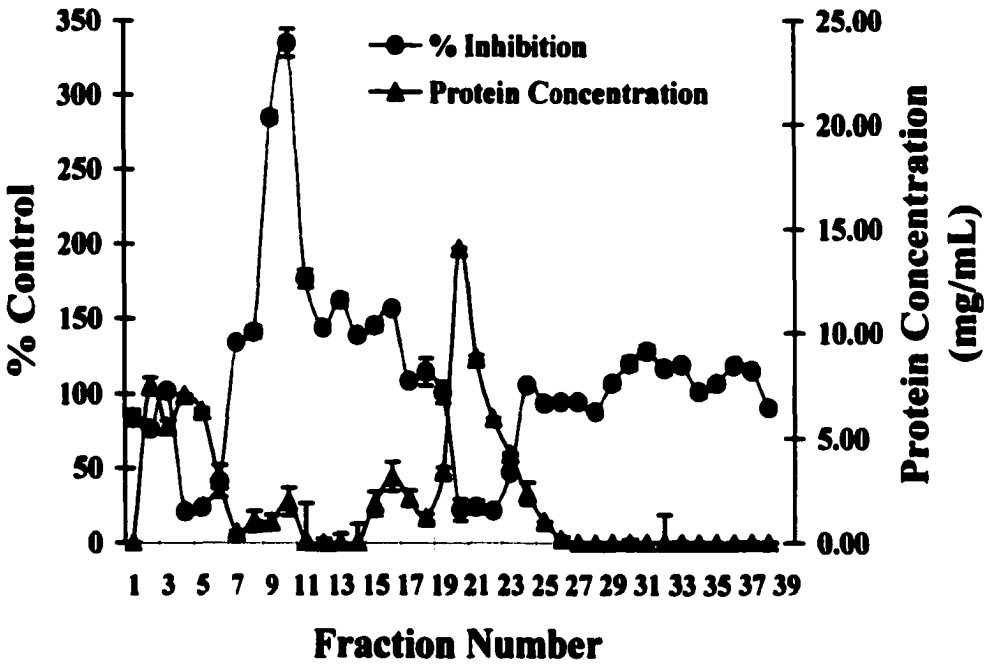

Figure 26. Effect of HPLC-fractionated TAP on HL-60 human myelogenous leukemia cell proliferation. Cultured HL-60 cells (20 x 10^3 cells/well) were exposed to either vehicle (complete RPMI-1640) or different fractions of HPLC separated TAP for 72 hr, and cell proliferation was determined by the MTT viability assay. Protein concentrations of individual fractions were determined with the Lowry assay. Data for HL-60 cell proliferation are expressed as the mean ±SEM of groups consisting of four observations.

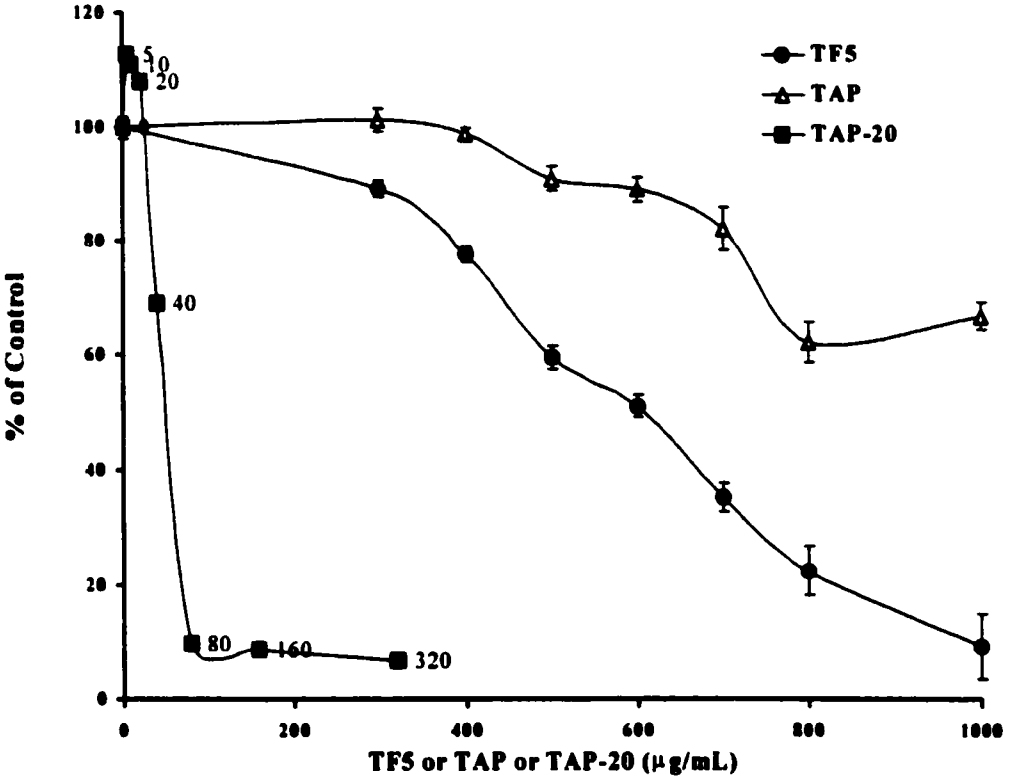

Figure 27. Effect of TAP fraction 20 on HL-60 cell proliferation: comparison to TAP and TF5. Cultured HL-60 cells (20 x 10^3 cells/well) were exposed to vehicle (RPMI-1640, supplemented) or TF5, TAP, or TAP fraction 20 for 72 hr and proliferation was measured by the MTT reaction assay. The data are expressed as the mean ± SEM of groups consisting of four observations.

REFERENCES

Asakawa M, Kinoshita Y, Kimura S, Hato F, Wada S, Nishijima T, Kishimoto T, Maekawa M (1990) Restorative effects of thymosin fraction 5 on reduction in responsiveness of rat thymocytes to allogeneic lymphocytes during bladder tumor induction. Cell Mol Biol; 36(3): 265-74.

Aspinall R (1997) Age-associated thymic atrophy in the mouse is due to a deficiency affecting rearrangement of the TCR during intrathymic T cell development. Journal of Immunology; 158(7): 3037-45.

Attia WY, Badamchian M, Goldstein AL, Spangelo BL (1993) Thymosin Stimulates Interleukin-6 Production from Rat Spleen Cells in Vitro. Immunopharmacology 26: 171-1796.

Badamchian M, Spangelo BL, Damavandy T, MacCleod RM, Goldstein AL (1991) Complete Amino Acid Sequence Analysis of a Peptide Isolated from the Thymus that Enhances Release of Growth Hormone and Prolactin. Endocrinology; 128(3): 1580-1588.

Badamchian M, Wang S-S, Spangelo BL, Damavandy T, Goldstein AL (1990) Chemical and biological characterization of MB-35: a thymic-derived peptide that stimulates the release of growth hormone and prolactin from rat anterior pituitary cells. Progress in NeuroEndocrinImmunology; 3:258-265.

Belcher, AE (1993) Leukemia. In Blood Disorders, Mosby's Clinical Nursing Series, Mosby: St. Louis, MO.

Berthiaume F, Aparicio CL, Eungdamrong J, Yarmush ML. (1999) Age- and disease-related decline in immune function: an opportunity for thymus-boosting therapies. Tissue Engineering Dec; 5(6): 499-514.

Bodey B, Bodey B Jr, Siegel SE, Kaiser HE. (2000) The role of the reticulo-epithelial (RE) cell network in the immuno-neuroendocrine regulation of intrathymic lymphopoiesis. Anticancer Research May-Jun; 20(3A): 1871-88.

Brelinska R, Warchol JB (1982) Receptors for thymosin fraction V on rat thymic lymphocytes. Experientia 38(5): 618-19.

Cohen GH, Hooper JA, Goldstein AL (1975) Thymosin-induced differentiation of murine thymocytes in allogeneic mixed lymphocyte cultures. Annals of the New York Academy of Sciences Feb 28; 249:145-53.

Cohen MH, Chretien PB, Ihde DC, Fossiek BE Jr, Makuch R, Bunn PA Jr, Johnston AV, Collins SJ, Gallo RC, Gallagher RE (1977) Continuous growth and differentiation of human myeloid leukaemic cells in suspension culture. Nature Nov; 270: 347-349.

Cowan MJ, Fujiwara P, Wara DW, Ammann AJ (1981) Effect of Thymosin on Cellular Immunity in Old Age. Mechanisms of Ageing Developments Jan; 15(1): 29-39.

Shackney SE, Matthews MJ, Lipson SD, Kenady DE, Minna JD; (1979) Thymosin fraction V and intensive combination chemotherapy. Prolonging the survival of patients with small-cell lung cancer. Journal of the American Medical Association Apr; 241(17): 1813-5.

Cupissol D, Rey A, Goldstein AL, Serrou B (1982) Decrease of tumor growth in mice after intravenous thymosin-treated bone marrow cell injection. Journal of the National Cancer Institute Jul; 69(1): 27-31.

Dalton WT Jr, Ahearn MJ, McCredie KB, Freireich EJ, Stass SA, Trujillo JM (1988) Concise Report: HL-60 Cell Line Was Derived From a Patient With FAB-M2 and Not FAB-M3. Blood Jan; 71(1): 242-247.

Dauphinee MJ and Talal N, (1975) Reversible restoration by thymosin of antigen-induced depression of spleen DNA synthesis in NZB mice. Journal of Immunology Jun; 114(6): 1713-6.

Dauphinee MJ, Talal N, Goldstein AL, White A (1974) Thymosin Corrects the Abnormal DNA Synthetic Response of NZB Mouse Thymocytes. Proceedings of the National Academy of Sciences, USA Jul; 71(7): 2637-2641.

Diezel W, Rentz E, Ermolin G, Sakandelidze OG, Sonnichsen N (1984) Augmentation of mitogen-induced interferon production in lymphocytes by thymosin. Experimental and Clinical Endocrinology Jul (1): 99-104.

Dillman RO, Beauregard J, Royston I, Zavanelli MI (1987) Phase II Trial of Thymosin Fraction 5 and Thymosin Alpha 1. Journal of Biological Response Modifiers, 6(3): 263-67.

Dillman RO, Beauregard JC, Zavanelli MI, Halliburton BL, Wormsley S, Royston I (1983) In Vivo Immune Restoration in Advanced Cancer Patients After Administration of Thymosin Fraction 5 or Thymosin α1. Journal of Biological Response Modifiers 2(2): 139-49.

Elfenbein GJ, Goldstein AL, Adams JS, Ravlin HM (1980) Thymosin-induced T cell marker expression and enhanced mitogen responsiveness in allogeneic transplant recipients Transplantation Feb; 29(2): 113-8.

Erbach GT, Bahr JM (1991) Enhancement of in Vivo Humoral Immunity by Estrogen: Permissive Effect of a Thymic Factor. Endocrinology Mar; 128(3): 1352-8;

Farah JM Jr, Hall NR, Bishop JF, Goldstein AL, O'Donohue TL (1987) Thymosin fraction 5 stimulates secretion of immunoreactive beta-endorphin in mouse corticotropic tumor cells. Journal of Neuroscience Research; 18(1): 140-6.

Forger JM, Cerny J (1976) Thymic hormone modulation of leukemogenic virus replication. Cancer Research 1976; 36(6): 2048-52.

Gallagher R, Collins S, Trujillo J, McCredie K, Ahearn M, Tsai S, Metzgar R, Aulakh G, Ting R, Ruscetti F, Gallo R (1979) Characterization of the Continuous, Differentiating Myeloid Cell Line (HL-60) From a Patient With Acute Promyelocytic Leukemia. Blood Sep; 54(3): 713-730.

Goldstein AL, Thurman GB, Cohen GH, Hooper JA (1975) Thymosin: Chemistry, Biology, and Clinical Applications. In Biological Activity of Thymic Hormones; van Bekkum, DW, ed.; Kooyker Scientific Publications, Rotterdam, The Netherlands

Goldstein AL, Guha A, Zatz MM, Hardy MA, White A (1972) Purification and biological activity of thymosin, a hormone of the thymus gland. Proceedings of the National Academy of Sciences, USA, 69:1800-1803.

Goldstein, Slater, White (1966) Preparation, assay and partial purification of a thymic lymphocytopoietic factor. Proceedings of the National Academy of Sciences USA, 56: 1010.

Hadden EM, Malec P, Sosa M, Hadden JW (1992) Mixed interleukins and thymosin fraction V synergistically induce T lymphocyte development in hydrocortisone-treated aged mice. Cellular Immunology Oct 1; 144(1): 228-36.

Hardy MA, Zisblatt M, Levine N, Goldstein AL, Lilly F, White A (1971) Reversal by thymosin of increased Susceptibility of Immunosuppressed Mice to Moloney Sarcoma Virus. Transplantation Proceedings March; 3(1): 926-28.

Hazama S, Hirose K, Enokihara H, Saito K, Komatsu H, Furusawa S, Shishido H, Konishi K (1978) In vitro influences of thymosin (fraction-V) on human peripheral blood T lymphocytes. Journal of Medical Science, 5(2): 321-7.

Hirokawa K, McClure JE, Goldstein AL (1982) Age-Related Changes in Localization of Thymosin in the Human Thymus. Thymus Jan; 4(1): 19-29.

Ho AD, Ma DDF, Price G, Hoffbrand AV (1983) Effect of Thymosin and Phorbol Ester on Purine Metabolic Enzymes and Cell Surface Phenotype in a Malignant T-cell line (MOLT-3). Leukemia Research 7(6): 779-86.

Ho AD, Ma DDF, Price G, Hunstein W, Hoffbrand AV (1983a) Biochemical and Immunological Differentiation of Human Thymocytes Induced by Thymic Hormones. Immunology. 50(3): 471-6.

Hooper JA, McDaniel MC, Thurman GB, Cohen GH, Schulof RS, Goldstein AL (1975) Purification and Properties of Bovine Thymosin. Annals of the New York Academy Sciences, Feb; 249:125-44.

Hsia J, Sarin N, Oliver JH, Goldstein AL (1989) Aspirin and Thymosin Increase Interleukin-2 and Interferon-g Production by Human Peripheral Blood Lymphocytes. Immunopharmacology, 17(3): 167-73

Hu SK, Badamchian M, Mitcho YL, Goldstein AL (1989) Thymosin Enhances the Production of IL-1b by Human Peripheral Blood Monocytes. Lymphokine Research, 8(3): 203-14.

Hu SK, Low TL, Goldstein AL (1981) Modulation of Terminal Deoxynucleotidyl Transferase Activity by Thymosin. Molecular and Cellular Biochemistry, Dec; 41:49-58.

Ikemoto S, Kamizuru M, Hayahara N, Okamaoto S, Wada S, Kishimoto T, Maekawa M (1992) Thymus lymphocytes in uraemic rats and the effect of thymosin fraction 5 in vivo. Clinical Experiments in Immunology, Feb; 87(2): 220-3.

Ikemoto S, Kishimoto T, Wada S, Hayahara N, Maekawa M (1990) Thymus lymphocytes in uraemic rats and the effect of thymosin fraction 5 in vitro. Clinical Experiments in Immunology, Jun; 80(3): 474-7.

Ikemoto S, Sugimura K, Wada S, Yasumoto R, Yamamoto K, Kishimoto T (1999) Augmentation of antitumor activity of 5'-deoxy-5-fluorouridine by thymosin fraction 5 in mouse bladder cancer cells in vitro and in vivo. Cancer Letters Oct 18; 145(1-2): 1221-6.

Jarvis WD, Kolesnik RN, Fornari FA, Traylor RS, Gewirtz DA, Grant S (1994) Induction of apoptotic DNA damage and cell death by activation of the sphingomyelin pathway. Proceedings of the National Academy of Sciences, USA; 91:73-77.

Kendall MD (1991) Functional anatomy of the thymic microenvironment; Journal of Anatomy. Aug: 177:1-29.

Keusch GT, Cruz JR, Torun B, Urrutia JJ, Smith H Jr, Goldstein AL (1987) Immature Circulating Lymphocytes in Severely Malnourished Guatemalan Children. Journal of Pediatric Gastroenterology and Nutrition, Mar-Apr; 6(2): 265-70.

Khaw BA, Rule AH (1973) Immunotherapy of the Dunning leukaemia with thymic extracts; British Journal of Cancer July; 28:288-92.

Kimura S, Ozaki Y, Toyokawa T, Nishio S, Maekawa M, Goldstein AL, Kinoshita Y (1986) Marked Reduction in Percentage of Rosette-Forming Thymocytes of Rats During Induction of Bladder Tumors and Restorative Effect of Thymosin in Vitro. Journal of the National Cancer Institute Apr; 76(4): 661-7.

Kinoshita Y, Kimura S, Hashimura H, Toyokawa T, Goldstein AL (1986) New System Examining Biological Activity of Thymus Hormone Using Rosette Formation Assay of Rat Thymocytes. Cellular and Molecular Biology, 32(4): 391-7.

Klein, Goldstein, White (1966) Effects of the Thymus Lymphocytopoietic Factor. Annals of the New York Academy of Sciences USA 135, 485.

Klein, Goldstein, White (1965) Enhancement of in Vivo Incorporation of Labeled Precursors into DNA and Total Protein of Mouse Lymph Nodes After Administration of Thymic Extracts. Proceedings of the National Academy of Sciences, USA 53, 812.

Kouttab NM, Goldstein AL, Lu M, Lu L, Campbell B, Maizel AL (1988) Production of Human B and T cell Growth Factors is Enhanced by Thymic Hormones. Immunopharmacology Sep-Oct; 16(2): 97-105.

Lavastida MT, Goldstein AL, Daniels JC (1981) Thymosin Administration in Autoimmune Disorders. Thymus. Feb; 2(4-5):287-95.

Leichtling KD, Serrate SA, Sztein MB (1990) Thymosin Alpha 1 Modulates the Expression of High Affinity Interleukin -2 Receptors on Normal Human Lymphocytes. International Journal of Immunopharmacology, 12(1): 19-29

Low and Goldstein (1978) Structure and Function of Thymosin and Other Thymic Factors. In The Year in Hematology, Silber et al., eds, Plenum Publishing Corporation, FRG.

Low and Goldstein, 1984; Thymosins: Isolation, Structural Studies and Biological Activities. In Thymic Hormones and Lymphokines. Goldstein AL ed., Plenum Publishing Corporation, FRG.

Low TLK, Thurman GB, McAdoo M, McClure J, Rossio JL, Naylor PH, Goldstein AL (1979) The Chemistry and Biology of Thymosin. I. Isolation, Characterization, and Biological Activities of Thymosin α1 and Polypeptide β1 from Calf Thymus. Journal of Biological Chemistry, 254:981-986.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein Measurement with the Folin Phenol Reagent. Journal of Biological Chemistry 1951; 193:265-275.

McGillis JP, Hall NR, Goldstein AL (1988) Thymosin fraction 5 (TF5) stimulates secretion of adrenocorticotropic hormone (ACTH) from cultured rat pituitaries. Life Sciences, 42(22): 2259-68.

Murai T, Iwata H, Mori S, Hato F, Kimura S, Kinoshita Y, Wada S, Kishimoto T, Makino S, Oohara T, et al (1995) Thymosin Fraction 5 Does Not Influence

Urinary Tract Carcinogenesis by Phenacitin and N-butyl-N-(4-hydroxybutyl) Nitrosmine in NON/Shi Mice. Oncology Research, 7(3-4): 139-44.

Mutchnik MG, Appelman HD, Chung HT, Aragona E, Gupta TP, Cummings GD, Waggoner JG, Hoofnagle JH, Shafritz DA (1991) Thymosin Treatment of Chronic Hepatitis B: A Placebo-Controlled Pilot Trial. Hepatology, Sep 14(3): 409-15.

Mutchnik MG, Schaffner JA, Prieto JA, Weller FE, Goldstein AL (1983) Increased Thymic Hormone Responsive Suppressor T Lymphocyte Function in Chronic Active Hepatitis. Digest of Disease Science, Apr; 28(4):328-34.

Naylor PH, Bhakoo HS, Paolini NS, Goldstein AL, Rosen F (1983) Immunomodulation of Murine B16 Melanoma Metastasis: Thymosin, Thymectomy and Irradiation. Thymus, Sep; 5(5-6) 355-61.

Neta R, Schwartz GN, MacVittie TJ, Douches SD (1985) Thymic hormones in radiation-induced immunodeficiency. I. Induction of mature interleukin 1 responsive cell in the thymus by thymosin fraction 5. Cellular Immunology, Sep 94(2): 480-90.

Neta R, Schwartz GN, MacVittie TJ, Douches SD (1985a) Thymic Hormones in Thymus Recovery from Radiation Injury. Experimental Hematology Today 14:6-13.

Oates, Sztein and Goldstein (1989) Mechanism of action of the thymosins: modulation of lymphokines, receptors, and T-cell differentiation antigens. Immunology Series; 45:273-88.

Okimoto T, Kinoshita Y, Hato F, Toyokawa T, Kimura S, Kinoshita H (1988) Effects of thymosin on soybean lectin responsiveness of thymic small lymphocyte subsets

from streptozotocin-induced diabetic rats. Cellular and Molecular Biology, 34(5): 465-72.

Pazmino NH, Ihle JN, Goldstein AL (1978) Induction in Vivo and in Vitro of Terminal Deoxynucleotidyl Transferase by Thymosin in Bone Marrow Cells from Athymic Mice. Journal of Experimental Medicine 1978 Mar; 147(3): 708-18.

Petro TM, Chien G, Watson RR (1982) Alteration of cell-mediated immunity to Listeria monocytogenes in protein-malnourished mice treated with thymosin fraction V. Infectious Immunology, 37(2): 601-8.

Petro TM, Watson RR (1982a) Resistance to L1210 Mouse Leukemia Cells in Moderately Protein-Malnourished BALB/c Mice Treated in Vivo with Thymosin Fraction V. Cancer Research, Jun; 42(6): 2139-45.

Raven and Johnson, (1992) The Immune System. In Biology, 3rd ed., Mosby Year Book Incorporated, St. Louis, MO.

Ruscetti FW, Collins SJ, Woods AM, Gallo RC (1981) Clonal Analysis of the Response of Human Myeloid Leukemic Cell Lines to Colony-Stimulating Activity. Blood, Aug; 58(2): 285-92.

Salvin SB, Neta R (1983) Resistance and Susceptibility to Infection in Inbred Murine Strains. I. Variations in the Response to Thymic Hormones in Mice Infected with Candida Albicans. Cellular Immunology, 75(1): 160-72.

Schafer LA, Goldstein Al, Gutterman JU, Hersh EM (1976) In Vitro and In Vivo Studies With Thymosin in Cancer Patients. New York Academy of Sciences, 277(00): 609-20.

Scheinberg MA, Goldstein AL, Cathcart ES (1976) Thymosin Restores T Cell Function and Reduces the Incidence of Amyloid Disease in Casein-Treated Mice. The Journal of Immunology, Jan; 116(1): 156-58.

Scher HI, Shank B, Chapman R, Geller N, Pinsky C, Gralla R, Kelsen D, Bosl G, Golbey R, Petroni G, et al (1988) Randomized Trial of Combined Modality Therapy with and without Thymosin Fraction V in the Treatment of Small Cell Lung Cancer. Cancer Research, Mar 15; 48(6): 1663-70.

Schulof RS, Low TL, Thurman GB, Goldstein AL (1981) Thymosins and other hormones of the thymus gland. Progress in Clinical and Biological Research; 58:191-215.

Schulof RS, Lloyd MJ, Ueno WM, Green LD, Stallings JJ (1984) Phase II trial of Thymosin Fraction 5 in Advanced Renal Cancer. Journal of Biological Response Modifiers, 3(2): 151-59.

Schulof RS, Simon GL, Sztein MB, Parenti DM, DiGioia RA, Courtless JW, Orenstein JM, Kessler CM, Kind PD, Schlesselman S, et al (1986). Phase I/II trial of thymosin fraction 5 and thymosin alpha one in HTLV-III seropositive subjects. Journal of Biological Response Modifiers. Oct; 5(5): 429-43.

Schultz RM, Papamatheakis JD, Stylos WA, Chirigos MA (1976) Augmentation of Specific Macrophage-Mediated Cytotoxicity: Correlation with Agents which Enhance Antitumor Resistance. Cellular Immunology, 25(2): 309-16.

Schurmann HJ, Van de Wijngaert FP, Delvoye L, Broekhuizen R, McClure JE, Goldstein AL, Kater L (1985) Heterogeneity and Age Dependency of Human Thymus Reticulo-Epithelium in Production of Thymosin Components. Thymus, 7(1): 13-23.

Serrate SA, Schulof RS, Leondaridis L, Goldstein Al, Sztein MB (1987) Modulation of Human Natural Killer Cell Cytotoxic Activity, Lymphokines Production, and Interleukin 2 Receptor Expression by Thymic Hormones. Journal of Immunology, Oct 1; 139(7): 2238-43.

Serrou B, Rosenfeld C, Caraux J, Thierry C, Cupissol D, Goldstein A (1979) Thymosin modulation of suppressor function in mice and man. Annals of the New York Academy of Sciences, 332:95-100.

Skotnicki AB, Zatz M, Sztein MB, Goldstein AL, Schulof RS (1988) Effect of Thymic Hormones on Interleukin 2 Synthesis by Lymphocytes from HIV-Positive Pre-AIDS Subjects. Immunological Investigations Apr; 17(2): 159-64.

Spangelo, BL (1995) Commentary: The Thymic-Endocrine Connection. Journal of Endocrinology, 147: 5-10.

Spangelo BL and Gorospe WC (1997) Thymic polypeptides and their role as mediators in neuroendocrine-immune communication. In Stress, Stress Hormones and the Immune System, Buckingham et al, eds., John Wiley & Sons, Ltd.

Spangelo BL, Farrimond DD, Pompilius M, Bowman KL (2000) Interleukin 1β and Thymic Peptide Regulation of Pituitary and Glial Cell Cytokine Expression and Cellular Proliferation. Annals of the New York Academy of Sciences, 917 (Neuroimmunomodulation): 597-607.

Spangelo BL, Farrimond DD, Thapa M, Bulathsinghala CM, Bowman KL, Sareh A, Hughes FM Jr, Goldstein AL, Badamchian M (1998) Thymosin Fraction 5 Inhibits the Proliferation of the Rat Neuroendocrine MMQ Pituitary Adenoma and C6 Glioma Cell Lines in Vitro. Endocrinology, 139(4): 2155-2162.

Sztein MB, Serrate SA, Goldstein AL (1986) Modulation of Interleukin 2 Receptor Expression on Normal Human Lymphocytes by Thymic Hormones. Proceedings of the National Academy of Sciences, USA Aug; 83(16): 6107-11.

Sztein MB, Serrate SA (1989) Characterization of the Immunoregulatory Properties of Thymosin Alpha 1 on Interleukin-2 Production and Interleukin-2 Receptor Expression in Normal Human Lymphocytes. International Journal of Immunopharmacology, 11(7): 789-800.

Sztein MB, Simon GL, Parenti DM, Scheib R, Goldstein AL, Goodman R, DiGioia R, Paxton H, Skotnicki A, Schulof RS (1987) In Vitro Effects of Thymosin and Lithium on Lymphoproliferative Responses of Normal Donors and HIV Seropositive Male Homosexuals with AIDS-Related Complex. Clinical Immunology and Immunopathology Jul, 44(1): 51-62.

Tabata T, Kinoshita Y, Fuji S, Hato F, Toyokawa T, Kimura S, Morii H (1989) Protection by Thymosin fraction 5 from Streptozotocin-Induced Diabetes in Mice. Cellular and Molecular Biology, 35(2): 121-7.

Thurman GB, Seals C, Low TL, Goldstein AL; (1984) Restorative Effects of Thymosin Poly- Peptides on Purified Protein Derivative-Dependent Migration Inhibition Factor Production by the Peripheral Blood Lymphocytes of Adult Thymectomized Guinea Pigs. Journal of Biological Response Modifiers, 3(2): 160-73.

Tijerina M, Gorospe WC, Bowman KL, Badamchian M, Goldstein AL, Spangelo BL (1997) A Novel Thymosin Peptide Stimulates Interleukin-6 Release from Rat C6 Glioma Cells in Vitro. Neuroimmunomodulation, May-Jun; (3): 163-70.

Wada S, Kinoshita Y, Ozaki Y, Nishio S, Kimura S, Maekawa M (1987) Reduction in responsiveness of thymocytes to phytohemagglutinin during bladder tumor induction and restorative effects of thymosin fraction 5 in rats. Journal of the National Cancer Institute, Feb; 78(2): 303-6.

Wara DW, Amman AJ (1976) Thymic Cells and Humoral Factors as Therapeutic Agents. Pediatrics, May; 57(5): 643-46.

Watson RR, Lim TS (1986) Thymosin Fraction 5: Effects on T Cell Functions in Mice Immunosuppressed by Severe Dietary Protein Deficiency. International Journal of Immunopharmacology, 8(6): 545-52.

Wiernik, PH, et al (1991) eds; Neoplastic Diseases of the Blood. 2nd ed, Churchill Livingstone Inc, NY.

Witherspoon RP, Hersman J, Storb R, Ochs H, Goldstein AL, McClure J, Noel D, Weiden PL, Thomas ED (1983) Thymosin Fraction 5 Does Not Accelerate Reconstitution of Immunologic Reactivity after Human Marrow Grafting. British Journal of Haematology, Dec; 55(4): 595-608.

Woyciechowska J, Goldstein AL, Driscoll B (1985) Experimental Allergic Encephalomyelitis in Guinea Pigs. Influence of Thymosin Fraction V on the Disease. Journal of Neuroimmunology, Jan; 7(4): 215-9.

Zatz MM, Goldstein Al (1985) Mechanism of Action of Thymosin I. Thymosin Fraction 5 Increases Lymphokine Production by Mature Murine T Cells Responding in a Mixed Lymphocyte Reaction. Journal of Immunology, Feb; 134(2): 1032-8.

Zatz MM, Oliver J, Goldstein AL, Novak C, Jacobs RP (1984a) Suppressor Cell Responses in Patients with Rheumatoid Arthritis: the Effect of Thymosin. Thymus, 6(4): 205-18.

Zatz MM, Oliver J, Samuels C, Skotnicki AB Sztein AB, Goldstein AL (1984) Thymosin Increases Production of T-cell Growth Factor by Normal Human Peripheral Blood Lymphocytes. Proceedings of the National Academy of Sciences USA May; 81(9): 2882-5.

Zatz MM, Skotnicki A, Bailey JM, Oliver JH, Goldstein, Al (1985a). Mechanism of Action of Thymosin II. Effects of Aspirin and Thymosin on Enhancement of IL-2 Production. Immunopharmacology, Jun; 9(3): 189-98

Zatz, MM, Oliver J, Sztein MB, Skotniki AB, Goldstein AL (1985b) Comparison of the Effects of Thymosin and other Thymic Factors on Modulation of Interleukin-2 Production. Journal of Biological Response Modifiers, Aug; 4(4): 365-76.

VITA

Graduate College
University of Nevada, Las Vegas

Melissa Pompilius

Local Address:
612 Donner Street
Las Vegas, NV 89107

Education:
Bachelor of Science, Chemistry, 1998
Northern Arizona University, Flagstaff AZ

Special Honors and Awards:
First Place, Natural and Physical Sciences Platform Presentation, Graduate Research Forum. University of Nevada Las Vegas, Las Vegas, NV, April 2000.
Graduate Research Training Assistantship, University of Nevada, Las Vegas. 6/2000-8/2000
Graduate Research Training Assistantship, University of Nevada, Las Vegas. 6/1999-8/1999

Publications:
Bryan L. Spangelo, Derald D. Farrimond, Melissa Pompilius, and Kay-Lynn Bowman. Interleukin-1β and Thymic Peptide Regulation of Pituitary and Glial Cell Cytokine Expression and Cellular Proliferation. Annals of the New York Academy of Sciences, Vol 917, p 597-607, 2000.

Melissa Pompilius, Derald D. Farrimond, W. David Jarvis, and Bryan L. Spangelo. Anti-Proliferative Actions of Thymic Hormones on HL-60 Human Promyelocytic Leukemia Cells. Abstracts of The Endocrine Society's 82ND Annual Meeting, Toronto, Canada, June 2000.

Thesis Title: The Effects of Thymic Hormones on the Proliferation of Human Myelogenous Leukemia Cells

Thesis Examination Committee:
Chairperson, Dr. Bryan Spangelo, Ph.D.
Committee Member, Dr. Ronald Gary, Ph.D.
Committee Member, Dr. Lydia McKinstry, Ph.D.
Graduate Faculty Representative, Dr. Steen Madsen, Ph.D.