The Effects of a Tibetan Medicinal Compound "Pokar 10" on the NIH 3T3 FIBROBLAST Cell line

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1. ABSTRACT

This experiment, conducted in Spring of 2004, at the Portland Community College, Rock Creek Campus, in the context of the Tissue Culture class of the Biotechnology Program, BIT 217, was designed with the purpose of exploring the possible effects of the Tibetan Medicinal Compound, Pokar-10, on the NIH - 3T3 Fibroblast Cell line. The cells, raised to near confluence in normal conditions, were transferred to two 96-well plates, and once properly settled, were then submitted to a nutritionally impoverished environment over eight days. They were given varying concentrations of the Medicinal Compound and observed microscopically for any visible responses. Their proliferation rate was monitored by means of an Analytical assay.

The Hypothesis proposing that the Tibetan Medicine (TM) would favorably influence the 3T3's cell cycle behavior by possibly accelerating cell division and thus enhancing their proliferation rate was supported. The results of the cell Proliferation Assay can be found in **Table1. & Figure1.** In addition, the cells showed apparent morphological changes in response to their unique interaction with the Medicinal Compound, indicative of its chemotropic properties. *Please refer to digital microscopic pictures.*

2. INTRODUCTION

BACKGROUND

The NIH-3T3 cell line, a standard Fibroblast line, is widely used in Western Research and industrial Biomedical applications. The line was established in 1962, from Swiss mouse *(Mus musculus)* Mesodermal embryonic tissue. These cells have the capability of growing indefinitely, and therefore are referred to as a continuous line (Davidson). The Fibroblast cells are precursor cells to Connective Tissue formation, and have the ability to differentiate into Cartilage forming cells, or Chondroblasts, Collagen forming cells, or Collagenoblasts, and Bone forming cells, or Osteoblasts, to name a few. They are responsible for forming the fibrous tissues in the body such as tendons and ligaments, and the looser binding tissues. They produce and secrete the extra-cellular matrix, or surrounding environment of the cells within the Connective Tissue, and play an important role in Tissue Healing by forming a gliding surface for Epithelial cells to migrate upon (Tortora & Grabowski). This ability to support the growth of skin cells or Keratinocytes, is widely used around the world for making skin grafts for burn victims (Davidson). Much research is still being conducted in order to determine the numerous other functions of these cells (Orr).

The Tibetan Compound Pokar-10, comprised of the following medicinal Herbs and Minerals, *Commiphora camphora, Terminalia chebula, Terminalia belerica, Emblica officinalis, Cassia tora, Cannabis sativa, Saussurea lappa, Tinospora cordifolia, Veronica ciliata, and Mineral pitch,* (Tsarong) is given in conjunction with other Tibetan pills, for the treatment of Joints, Connective Tissue Inflammation and pain, Blood and Serum disorders, as well as Skin problems (Namdul). It was provided from the Men-Tsee-Khang, the Tibetan Medical and Astrological Institute of His Holiness the Dalai Lama, in *Dharamsala*, India.

As the preliminary response of cells were observed microscopically for morphological and behavioral changes over a four-day period, the most favorable concentrations of Tibetan Medicine were then determined for further testing procedures *(please refer to **Plate #1** of Materials & Methods below)*. The analytic procedure of this experiment, a Proliferation Assay using Tetrazolium salt (MTS) as reagent dye in a chromogenic reaction, was carried out over four days in order to analyze the different proliferative responses of the NIH - 3T3 cell line in the presence of two concentrations of TM, all while being submitted to an impoverished medium *(please refer to **Plate #2** of M & M below)*.

The cells were read by Spectrophotometry, measuring of the amount of light absorbed by the stained cytoplasmic material, the intensity of the color absorbed at a peculiar wavelength (here A_{490} nanometers used) being directly proportional to the cell population within the well. Two readings where done at a 30-hour interval, the first reading taken almost two days from the time of the set-up in order to allow the cells enough time to respond to their environmental conditions.

HYPOTHESIS

The Hypothesis of this experiment suggested that the implementation of the Tibetan Medicinal Compound, Pokar-10, ordinarily given for the treatment of joints & Connective Tissue inflammation, would be beneficial in some way to the Fibroblasts, precursor cells to Connective Tissue formation.

The TM Pokar-10 could possibly influence the 3T3's cell cycle by accelerating the cells' Proliferation rate, which would be verifiable by the analytic assay. Other outcome markers for measuring the Compound's impact would include cell observation for morphological changes, such as in size or shape, changes in cell survival time compared along controls, and/or varying responses to maximum confluence. These predilections would be based on the assumption that any of the behavioral responses of the cells would be visible on the Phase Contrast Microscope.

3. MATERIALS AND METHODS

NIH –3T3 Fibroblast cells_____ derived from Swiss Mouse embryos and ordered from the American Type Culture Collection, (ATCC) were grown to 75% Confluence in a T_{75} Tissue Culture flask, at Passage P_{34} , by EH on 05/14 /04 and seeded by operator CRP on 05/17/04 at a density of 1x 10^3 cells in a volume of 100 uL per well, occupying 60 out of the 96 wells, in two 96 well-plates (Corning), at Passage P_{35}. Each plate is comprised of twelve columns of wells, numbered 1 through 12, and eight rows, lettered A through H.

In each plate (Corning), the outer-border wells were filled with 200 uL of autoclaved Milli-Q or de-ionized H_2O in order to avoid evaporation of the small volume samples. The different conditions being tested were plated in columns 2 through 11, and in rows B through G of the plates (row A thru G for plate #2), (*please refer to tables of Plates 1 & 2 below for exact set-up)*. The same multi-channel micro-pipettor (JENCONS) was used throughout the platting for accuracy. All handling and sub-culturing of cells were done in sterile conditions under sanitized Tissue Culture hoods. (slide no 9)

Regular Tissue Culture medium _____was prepared with two different calf serum concentrations. The Dulbecco's Modified Eagle Medium, or DMEM (GIBCO Laboratories, Grand Island, N.Y.), is a standard commercial medium containing:

- 15 Amino acids, *(L-Arginine, L-Cystine, L-Glutamine, Glycine, L-Histidine, L-Isoleucine, L-Leucine, L-Lycine.HCl, L-Methionine, L-Phenylalanine, L-Serine, L-Threonine, L-Tryptophan, L-Tyrosine.2Na.2H2O, L-Valine)*
- 6 Inorganic salts, *(Calcium Chloride, Potassium Chloride, Sodium Chloride, Sodium Phosphate, Ferric Nitrate, & Magnesium sulfate)*
- 8 Vitamins, *(Choline Chloride, Folic acid, Myo-Inositol, Niacinamide, D-Panthothenic acid, Pyridoxal.HCl, Riboflavin, Thiamine.HCl)*
- D-Glucose and Pyruvic Acid.Na

The first DMEM preparation was supplemented with **10% Bovine Calf Serum, BCS** (GIBCO Calf Serum Lot# 39070), medium in which the 3T3s ordinarily thrive. The second DME medium was supplemented with **only 1% BCS**, stressing the cells considerably. In both cases an Anti-fungal, Fugizone at a concentration of [1.25 ug (microgram) /mL (milliliter)] and the Antibiotics, Penicillin/Streptomycin [100ug/mL] were added.

The Tibetan Medicinal Compound, Pokar-10_____ was provided in the form of round capsule-less pills of tightly packed dried ingredients. The pills were ground in a coffee-bean grinder, Model CG-2 (Melita) and further crushed into a fine powder using a pestle and mortar. Three stock solutions of a volume of 1 mL each of concentrations of: [3 mg/mL], [2.4 mg/mL] and [1 mg/mL] were prepared in 1.5 mL microfuge tubes by diluting the powder into near boiling autoclaved MQ H_20, and by vortexing continuously for 30 to 40 minutes, to best dissolve the insoluble components of the medicine. The tubes were stored several hours at room temperature (RT), until use.

The volumes needed for the desired concentrations of [100 ug/mL], [10 ug/mL] and [1ug/mL] of suspended TM dissolved in DMEM, 1% BCS, were prepared by adding the appropriate volume of medium to a re-vortexed TM stock solution just before use.

Plate # 1_____ The purpose of this set-up was to determine the most suitable concentration of TM for the amount of cells seeded in the small volumes (100 uL) within the wells. The plate was seeded on 05/17 /04 at one thousand 3T3 cells / well, in a 96-well-plate, and the cells were submitted to the following conditions on 05/18 /04:

Row A. Columns 1 through 12, 200 uL of MilliQ water only
Row B. Control, **CTRL**, Col. 2- 11, 3T3s in DMEM with 10% BCS, no TM
Row C. **CTRL,** Col. 2- 11 3T3s in DMEM with 1% BCS, no TM
Row D. Col. 2- 11, 3T3s in 1% BCS and **1ug / mL** of TM
Row E. Col. 2- 11, 3T3s in 1% BCS and **10 ug / mL** of TM
Row F. Col. 2- 11, 3T3s 1% BCS and **100 ug / mL** of TM
Row G. **CRTL,** Col. 2- 11, 3T3s **10% BCS** and **300 ug / mL** of TM
Row H. Columns 1 through 12, 200 uL of MilliQ water only

Plate # 2 _____This plate was prepared for the Analytical assay in order to monitor the cells' proliferative response to the TM by recording their density within the wells over time. The cells were treated with the reagent dye MTS (Tetrazolium salt, [2 mg/mL] ,*Promega*) in a chromogenic reaction, staining their cytoplasmic contents.

Following the manufacturer's instructions, a volume of 20 uL of reagent was added per well being analyzed, and the plate was left to incubate for 2 hours in 5% CO_2 , at 37° Celsius. The wells treated with MTS were then read at A_{490}, (wave-length: 490 nm) on the Bio-Tek 96 well-plate Spectrophotometer. The wells yet to be treated were protected by a Parafilm cover during the Spect reading. The plate was seeded on 05/17 /04 at one thousand 3T3 cells per well, in a 96-well plate, and the cells were submitted to the following conditions on 05/21 /04:

Row H. Columns 1 through 12, 200 uL of MilliQ water only
Row G. Control, **CTRL**, Col. 2- 11, 3T3s in DMEM with 10% BCS, no TM
Row F. **CTRL,** Col. 2- 11 3T3s in DMEM with 1% BCS, no TM
Row E. Col. 2- 11, 3T3s in 1% BCS and **5 ug / mL** of TM
Row D. Col. 2- 11, 3T3s in 1% BCS and **5 ug / mL** of TM
Row C. Col. 2- 11, 3T3s 1% BCS and **10 ug / mL** of TM
Row B. Col. 2- 11, 3T3s 10% BCS and **10ug / mL** of TM
Row A. **CTRL,** Columns 1 through 12, **No cells** and **10ug / mL** of TM

and read twice on Spectrophotometer, at 64h 20 min., and 94h 20 minutes thereafter.

4. RESULTS

In order to determine the most suitable concentrations of Pokar-10, the 3T3 cells' metabolic behavior & response to the TM were observed daily under the Phase Contrast microscope for the detection of any visible changes.

On 5/ 20 / 04, after three days of treatment in deprived medium, the cells submitted to smaller concentrations of TM, notably [10 ug/mL] and [1ug/mL], were noticed to be growing "extensions" of their cytoplasmic material, appearing to be reaching specifically for the Tibetan Compound, grabbing and engulfing bits of it. This movement in the direction of the TM can be referred to as a positive chemotropic response.

Some illustrations of this behavior is exhibited on the Microscopic pictures taken on 5/21 /04 at the Primate Center of the Oregon Health and Science University, OHSU West Campus, obtained using a digital camera mounted onto a Phase Contrast Microscope, *opportunity graciously provided by our Instructor, Kalama Taylor, M.S., Core Tissue Culture plant,* OHSU.

The larger concentrations of [100 ug/mL] & ([300 ug/mL] Control) seemed to be overpowering to the 3T3s, filling the contents of the wells and literally masking the presence of cells. Also the cells died out rather quickly, after three to four days (Data not shown).

A second set of pictures were taken on 5/ 25 / 04, eight days after the beginning of the experiment, (see slide 10 #511 of **Plate # 1**), where all the cells clearly appeared necrotic at this point. The two slides 12 #515, & 13 #517 of **Plate # 2** also demonstrated cell death occurring after 4 days of treatment. Here the cells were given a DME + 1% BCS medium with TM concentration of [5 ug/mL] and [10 ug/mL], however this time, the insoluble Medicinal components were removed by sterile filtration before being given to the cells.

A 96-well Assay using MTS as reagent dye, was conducted over nearly a four-day period, in order to analyze the proliferative response of NIH – 3T3 Fibroblast cells when submitted to two different concentrations, respectively [5 ug/mL] and [10 ug/mL] of Pokar-10, as compared to a control which was not given the medicine. The results represented in ***Figure 1.*** illustrate the graphic representation of the Assay data, mean of Absorbance values at 490 nanometers versus Time, and comparing the conditions side by side, first at 64 h 20 min., then at 94 h 20 min.

It appeared that the cells that were given the Tibetan Compound underwent double (for 10 μg/mL) and even triple (for 5 μg/mL) the amount of proliferation as compared to the Control cells. This would suggest that Pokar-10 was influential in some way to the Cells' Cycle and Mitotic division. Each of the Absorbance readings at A_{490} obtained by means of Spectrophotometry, is represented in the corresponding data **Table 1.** ***Results of the Proliferation Assay***.

5. DISCUSSION

The results of this modest experiment seem to have supported the proposed Hypothesis, stating that the implementation of minute quantities of the Tibetan Medicinal Compound Pokar-10 would favorably affect the metabolic behavior and proliferation rate of a NIH - 3T3 Fibroblast Cell Line, when submitted to stressful conditions. Both the morphological changes observed, as well as the cell density analysis are supportive of this Hypothesis.

Normally cells cultured in 96-well plates require fresh and sterile filtered 10 % BCS medium every other day for their survival. The fact that some of the cells survived in 1% BCS medium implemented with TM beyond 72 hours, seemed remarkable in itself.

It was interesting to notice that the cells that received the first medium preparation containing the insoluble particles of TM in suspension (visible as the golden/brown specks on the digital slides) survived about double the time as compared to those that received sterile filtered TM supernatant only (as shown in slides 12 #515, & 13 #517). Further more, the surprisingly and apparent morphological adaptations of the cells demonstrating their attraction to the TM particles, as shown in slides 3 #482, 5 #483, & 7 #484, confirmed the medicinal Compound's chemotropic properties.

The cell Density Analysis was performed without a Time Zero, usually done within the first few hours of seeding the cells in the 96-well plate, in order to establish a base line for comparison. This would be useful to include in future studies. Considering that the implementation of [5 ug/uL] of TM almost tripled the proliferation rate of the cells over the control cells, the Hypothesis suggesting the possible prolific properties of the TM is supported. Subsequent studies with similar results would serve to be confirmatory.

Whether, and more rightfully *How~* the Medicinal Compound Pokar-10 aided in the cell's general Metabolic activities, duplication of the Genetic material DNA, duplication of the Organelles or the Cytosolic components within the Interphase of the Cell Cycle, and/or more directly in the process of Nuclear division, or Mitosis, is of course yet to be determined… Further Scientific experimentation and data collection would be warranted in order to continue documenting the responses of the 3T3 cells in the presence of Pokar-10, and in attempts to understand the precise Physiological mechanisms that were involved. Additionally, the molecular study of Pokar-10's herbal and mineral composition might be proven useful.

In closing, we are deeply thankful to the **Men-Tsee-Khang,** *Tibetan Medical and Astrological Institute of His Holiness the Dalai Lama*, in Dharamsala, and the Board of Tibetan physicians for having given us the permission to conduct this experiment on their Medicinal Compound. We stay hopeful that future scientific studies will bring to Tibetan Medicine the recognition it deserves.

6. REFERENCES

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