

# A Novel Human Hormone-peptide NEROFE™ with Strong anti Cancer Activity.

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**Abstract:** - We report the isolation of a novel Tumor-Cells Apoptosis Factor (Nerofe™). We found that cDNA of this protein is expressed mainly in the human thymus and partially in the colon and in the frontal lobe of brain. Immuno histochemical studies localize Tumor-Cells Apoptosis Factor 's (TCApF) to the medulla and Hassal's corpuscles of the thymus gland, which are responsible for the negative selection. It has been found in that TCApF is a naïve ligand of T1/ST2 receptor. This fact together with sequence analysis and presence of free TCApF in blood samples allow considering TCApF as a hormone-like protein. Application of TCApF and its short derivative TCApFs to acute myeloid leukemia proliferating cells (U937 premyeloid cells), human breast carcinoma (MCF7), human glioblastoma, human prostate cancer, murine mammary adenocarcinoma and human lung cancer proliferating cells inhibits the proliferation *in vitro*. In contrast, TCApF doesn't affect the healthy cells. Treatment of mice with induced AML terminates the cancer development and completely eliminates metastasis cell colonies from the bone marrow and the spleen that reduces probability of the cancer return.

**Key-Words:** Hormone-peptide, AML drugs, cancer, biomarker.

## 1 Introduction

An idea to use naïve ligands of the death receptors, like TNF and TRAIL, as promising drug compounds for the cancer treatment, has become quite popular in the last decades [1-4].

In this paper we report about novel ligand of T1/ST2 receptor - Nerofe™ (Tumor-Cells Apoptosis Factor - TCApF). A member of the Toll/Interleukin receptor superfamily - T1/ST2 receptor has been known more than 20 years. Originally, this receptor was discovered as an early response gene following over-expression of the *v-mos* or *Ha-ras* oncogenes [5]. In spite the fact that T1/ST2 is highly expressed in several human cancers such as breast carcinoma, NSCLC cells and AML cells [6-7], its role in cancer cells remained an enigma. We have found that T1/ST2 is a selective death receptor for proliferating cancer cells and TCApF is naïve ligand of T1/ST2. Binding TCApF to T1/ST2 activates caspases 8 and Bcl-2 mediated downstreams in the cancerous cells, which highly express the receptor. These processes culminate in apoptosis of the proliferating cancer cells [8-9].

Acute myeloid leukemia (AML) is the most common type of blood cancer in adults. If untreated,

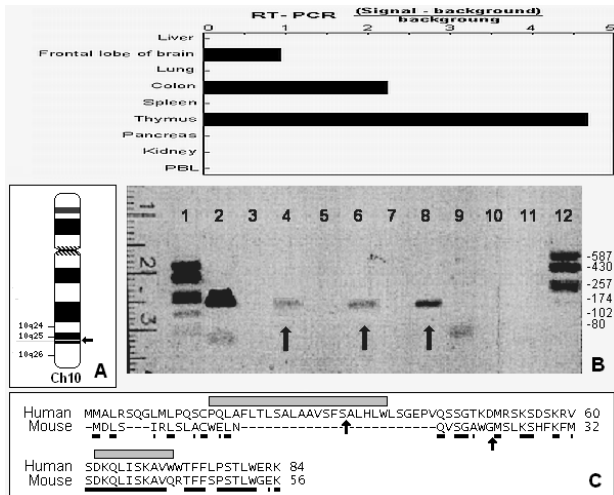
this form of leukemia progresses very quickly [13]. We will show that application of TCApF and its short derivative TCApFs to proliferating AML cells, selectively inhibits their proliferation, but doesn't affect the healthy cells (the same result was obtained for several other cancerous cell lines). Treatment of mice with induced AML terminates the cancer development and completely eliminates metastasis cell colonies from the bone marrow and the spleen. Toxicology study doesn't reveal considerable adverse effects up to 15×(Therapeutic Effective Dose) and mild adverse effects for dose 20×(Therapeutic Effective Dose).

## 2 Results

### 2.1 Cloning of TCApF gene and synthesis of the gene product

Screening the human genome with bioinformatics tools enabled us to find a sequence in chromosome 10 (HUM: 10q25.3; AL133482.15; GI: 15617233; region 16945...17199), which appears to encode an open reading frame for a protein with a signal peptide sequence at its N-terminus (Fig.1C). In the mouse genome the analog sequence was found in chromosome 19 (MUS:19; AC161579; GI:71143306; region 84125...84367).

RT-PCR screening of human cDNA libraries prepared from different tissues enabled us to identify a new cDNA mainly present in the human thymus cDNA library and partially in colon and frontal lobe of brain cDNA libraries, but not in other tissues. The sequence of the RT-PCR product was identical to the predicted human sequence.

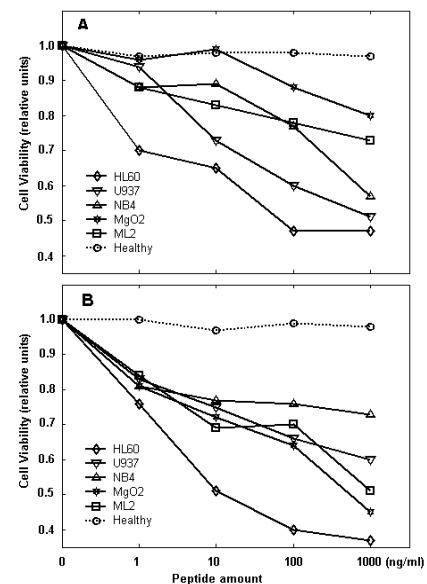


The human gene contains only one exon, which has initiation and termination codons and is encoding an 84 amino acid peptide (molecular weight 10.9112 kDa). Computational structural analysis of the deduced amino acid sequence revealed that the peptide has a signal sequence at positions 1... 33. It has been found in [8] that the peptide is a naïve ligand of T1/ST2 receptor. Predicted secondary structure of the peptide, its size, solubility, compactability and presence of the free peptide in blood imply that the peptide is a hormone-peptide. We named the the 51 amino acid peptide without the signal sequence Tumor-Cells Apoptosis Factor (TCApF, molecular weight 6.8796 kDa). We raised a polyclonal antibody against TCApF and used it for Immunological Histological Chemical studies (IHC) in different human tissue sections. The results of the IHC show that TCApF is expressed in the thymus medulla and the thymus Hassall's corpuscles, but was there no staining in the thymus cortex. Other

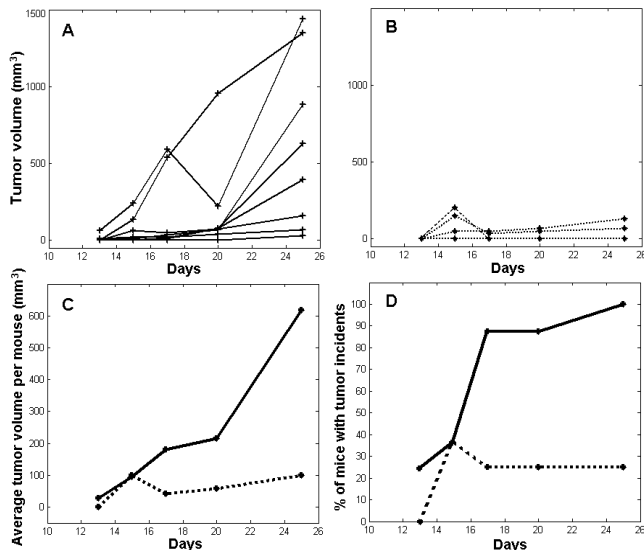
lymphatic lobes, spleen and liver sections did not bind the antibody. These results confirm the RT-PCR results. It should be noted that the staining appears in the cell's cytoplasm as expected for a secreted peptide. The cells expressing TCApF were identified as fibroblasts. The mouse gene encodes 56 amino acids peptide with a signal sequence at positions 1... 23 (Fig.1C).

**2.2 TCApF inhibits proliferation of the cancer cells, but doesn't affect the healthy cells *in vitro*.**

We have applied TCApF to different lines of the AML cancer cells and found that TCApF inhibits their proliferation *in vitro* in a dose-dependent manner (Fig.2A). Note that the short derivative of TCApF, which contains 14 amino-acids, holds anti-cancer activity (Fig.2B). This short peptide was named TCApFs (molecular weight 2.1314 kDa). It was found also that TCApF as well as TCApFs inhibit proliferation of human breast carcinoma cells (MCF7), human glioblastoma cells, human prostate cancer cells and human lung cancer cells (not shown). It is important that TCApF and TCApFs don't affect proliferation of the healthy cells (Fig.2).



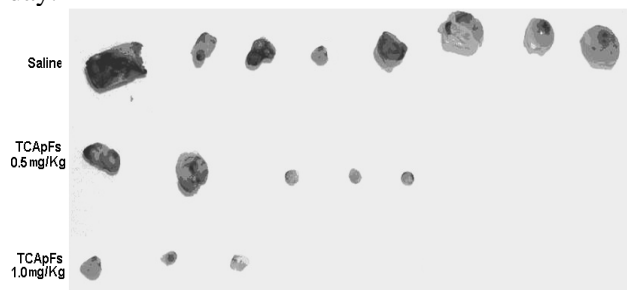
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**Fig.3.** Tumor growth over time in mice, which have been injected with NB4 human promyelocytic cells. **A** – mice treated with saline. **B** - mice treated with 1.0mg/Kg TCAPFs. **C, D** **Solid** lines - mice treated with saline, **dashed** lines - mice treated with 1.0mg/Kg TCAPFs.

### 2.3 TCAPFs suppress cancer development *in vivo* (animal model).

Animal model study is in good agreement with *in vitro* results. BALB/c nude mice were inoculated S.C. into the right flank of 8-week-old mice (10 mice for each treatment group) with U937 and NB4 cells. Since cancer cells were inoculated S.C., the solid tumor was developed together with acute leukemia. After 7 days solid tumor size was measurable and the mice were randomly divided into 3 groups (each one 8 mice). Mice of the control group were injected with saline, while the treated group mice were injected IP with 0.5mg/Kg and 1.0mg/Kg concentrations of TCAPFs each other day.



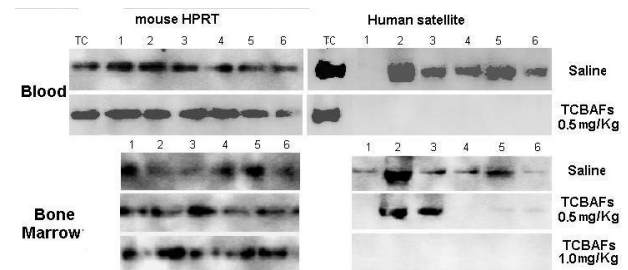
**Fig.4.** Solid tumor samples have been extracted from 3 groups of mice were treated with different doses of TCAPFs. Each group contains 8 mice. In the group was treated with 1.0mg/Kg TCAPFs only 3 mice slowly developed the tumor during 25 days, while in the other mice the tumor was eradicated.

Tumor growth was monitored using calipers every 2 or 3 days. Fig.3 shows the tumor size development

during 18 days after inoculation of the cancer cells. It is clear seen that both tumor size and cancer incidents were significantly lower in the group that was treated with 1.0mg/Kg concentration of TCAPFs in comparison with the control group. The tumor decreases in a dose dependent manner that is seen in Fig.4.

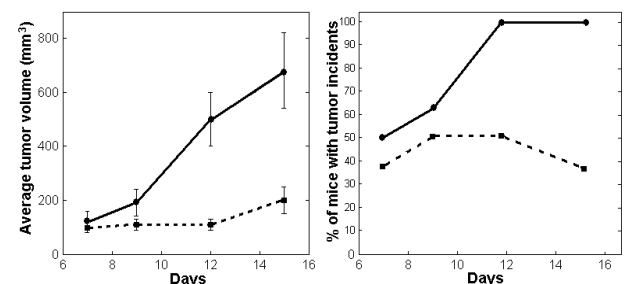
It should be noted that TCAPFs not only suppress the tumor grows, but also enables eradication of the developed tumor (Fig.3 and Fig.4).

One of a problem in AML treatment is that the cancer cells can develop colonies in the bone marrow and the spleen. These colonies are eliminated with difficulty, so cancer can return after chemotherapy. We have checked the influence of TCAPFs on these colonies by using RT-PCR for human satellites in mouse blood, bone marrow and spleen. In order that we be able indicate even one cancer cell, 40 cycles of PCR have been done.



**Fig.5** Results of RT-PCR analyses of blood and bone marrow of the mice, which have been injected with U397 human premyeloid cells. Mice were treated with saline (control group) and with different doses of TCAPFs. Mouse HPRT gene was used as a positive control. 40 cycles of RT-PCR have been done. TC – tumor cells, 1 ... 6 number of mouse. (Each experiment has been repeated at least 3 times).

The results are shown in Fig.5. It is clearly seen that a 1.0mg/Kg dose of TCAPFs completely eliminates the cancer cells from the bone marrow. The same results were obtained for the spleen (not shown).



**Fig.6.** Tumor grows in Balb/C mice have been injected S.C. with EMT6 murine carcinoma cells. **Solid** line – mice treated with saline. **Dashed** line - mice treated with 1.0mg/Kg TCAPFs.

In order to check that TCAPFs able to suppress not only an exogenous tumor, but a endogenous tumor

as well, we have applied TCApFs to mice with murine carcinoma. The results are shown in the Fig.6 and they are similar to those the shown in the Fig.3.

## 2.4 Toxicology study

Pharmokinetic study shows that the time required for a change the amount of TCApFs in the body by one-half during elimination ( $T_{1/2}$ ) is about 5h, so the peptide is quite stable.

Toxicology study was done by Harlan Laboratories Israel, Ltd. (ISO 9001:2000 Certificate No.: US2002/3081). Two treatment cycles, interspaced by 1-week interval, each confined to 1×daily repeated intravenous (IV) dosing sessions for 7 successive days, were done for each animal.

Most of the statistically significant mean group values of hematology and biochemistry analyses were found to be within the range of the reference values of the rodent species employed in the study. However, total bilirubin levels and AST & ALT levels of males subjected only to the High-dose (20 times more than the therapeutic effective dose) were found to be out of the range as well.

Histopathological examinations of the Vehicle Control (0 mg/Kg of TCApFs) and the High-dose groups indicated the presence of target organs in the liver, skeletal muscle and heart. In the liver, treatment-related changes were seen at a relatively comparable incidence and severity in both sexes, and consisted of minimal hepatocytic single cell necrosis, distributed at random and without any site predilection. This change was associated with increased incidence and severity (up to grade Mild) of inflammatory cell foci. In the skeletal muscle of 2 from 24 male rats, minimal multifocal myofiber cell degeneration associated with fibrosis was noted. In the heart of two male and three female rats, minimal myocardial degeneration, associated with fibrosis was noted. It should be noted that for Middle (15 times more of the therapeutic effective dose) and lower (10 times more of the therapeutic effective dose) doses no statistically significant changes have been found.

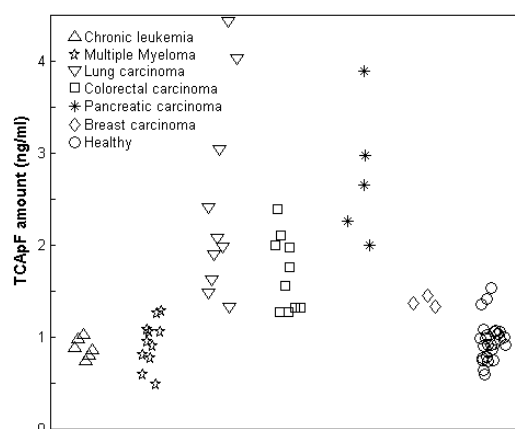
## 2.5 Diagnostic ability of TCApF

Since free TCApF is presented in human blood, we have checked the possibility of using this protein as a biomarker.

Fig.7 shows results of measuring of TCApF amount in blood samples of patients with different types of cancer: breast carcinoma, lung carcinoma, colorectal carcinoma, pancreatic carcinoma, chronic lymphocytic leukemia and multiple myeloma. For all

types of the carcinoma cancer the amount of TCApF ( $2.07 \pm 0.87$  ng/ml) is significantly higher than in the healthy individuals ( $0.94 \pm 0.25$  ng/ml). It is interesting that in the healthy male amount of TCApF was found somewhat higher than in the healthy female.

TCApF demonstrates high sensitivity ~100% and high specificity ~88% for carcinoma type cancer (no false negative response was found for 34 blood samples of the patients with known cancer and only 3 false positive response for 26 blood samples of the healthy persons). On the other hand there was not found significant difference between TCApF level in healthy persons and in patients with non-carcinoma cancer, like CLL or multiple myeloma (Fig.7).



**Fig.7.** Amount of free TCApF in samples of blood serum of the patients with different types of carcinoma and in blood samples of healthy persons. The healthy group includes 26 samples, with amount of TCApF= $0.94 \pm 0.25$  ng/ml. Total samples of the carcinoma type cancers is 34, with amount of TCApF= $2.07 \pm 0.87$  ng/ml.

## 3 Discussion

Multiple hard adverse effects are now one of the main problems in contemporary chemotherapy for elderly patients. For example, more than half of the elderly patients with AML are not eligible for intensive chemotherapy and are able to receive only conservative treatment [13]. The similar picture exists for many other types of cancer as well. So, search for effective anti-cancer drugs with no or minor adverse effects are a big challenge in chemotherapy nowadays.

We have isolated and cloned the novel human hormone-like protein Nerofe<sup>TM</sup> (TCApF - Tumor-Cells Apoptosis Factor). It was found [8] that TCApF is expressed mainly in the human thymus medulla and Hassall's corpuscles, and partially in the colon and the frontal lobe of brain. It was found also [8] that TCApF is a naïve ligand of T1/ST2

receptor [5,6,10]. It is interesting that the medulla and Hassall's corpuscles are responsible for negative selection process [11], while the frontal lobe of the brain is related to *in vivo* immune response [12].

Both *in vitro* and *in vivo* (mice animal model of AML) studies reveal strong anti-cancer activity of TCApF and its short derivative TCApFs. It is important that TCApF and TCApFs were able not only to suppress cancer development, but eradicate the tumor as well. It should be emphasized that 1.0mg/Kg dose of TCApFs was able to completely eliminate metastasis colonies from the bone marrow and the spleen and, thus, considerably reduce probability of the cancer return.

Toxicology study shows no considerable adverse effects up to 15×(Therapeutic Effective Dose) and mild adverse effects for dose 20×(Therapeutic Effective Dose).

Presence of free native TCApF in a human blood makes it possible to use this protein as a sensitive and specific biomarker for carcinoma type cancers.

## Materials and Method

### Peptide synthesis and polyclonal antibodies

TCApF and its FITC derivative were synthesized by ANASPEC Inc, San Jose, CA 95131. The peptide used for the experiments was 96% pure by HPLC. Polyclonal antibodies were raised by ANASPEC Inc, San Jose, CA 95131.

### Immuno Histo Chemistry

Three cases of human thymus were selected from the pathology archives. The specimens were formalin-fixed or fixed in B5 and paraffin-embedded. Sections (3 and 5 µm thickness) were obtained from representative blocks of paraffin embedded thymus from each patient. Controls were sectioned at 5 µm thickness. Sections were submitted to heat retrieval, blocking steps, then incubated with primary antibody, washed and incubated with biotin labeled secondary antibody, washed, incubated with substrate and counterstained. Sections were examined by bright field microscopy.

### RNA Harvesting and RT-PCR

RNA was harvested from cells using Promega Kit (EZ RNA) and cDNA was prepared using EZ cDNA kit (IBI Ltd). For PCR the following procedure was employed: 1 min 95°C; 40 cycles of: 1 min at 95°C; 1 min at 52°C; 1 min at 72°C. 10 min at 72°.

### Oligonucleotides

Oligonucleotides were synthesized by IDTDNA Ltd.

The sequence of oligonucleotides we used for the screening of the different cDNA libraries were:

5'- ATGGCACTCAGAAGCCAGGG -3'  
5'- CACTCGCTTGGGAATCGGATT -3'.

### Cell Lines

The human monocytic leukemia cell line U937 and the human acute promyelocytic leukemia cell line NB4 were grown in RPMI 1640 medium (Biological Industries, Kibbutz Beit Ha'emek, Israel) containing penicillin (100 units/ml), streptomycin (100 tgz/ml), amphotericin (0.25 /zg/ml), 10 /zM /3-mercaptoethanol, and 0.2% (w/v) glucose, together with 10% foetal calf serum (Biological Industries, Kibbutz Beit Ha'emek, Israel) (complete RPMI medium) in 5% CO<sub>2</sub>/humidified air at 37°C, in suspension culture in 260 ml plastic bottles (Nunc, Denmark).

### *In vitro* experiments

Cells were divided in 96 well plate (30000 cells/well). Chemicals were applied to cells in different concentrations for 24 hours. Following this incubation Resazurin was applied to cells for 4 hours. Absorbance was measured. Results represent quadruplicates. Each experiment has been repeated at least twice.

### *In vivo* experiments

BALB/c nude mice were purchased from Harlan (Jerusalem, Israel). Female nude mice were kept under a specific pathogen free (SPF) environment. All experiments were approved by the Institutional Animal Care and Use Committee. Mice were inoculated S.C. into the right flank of 8-week-old mice (10 mice for each treatment group) with 2×10<sup>6</sup> U937, NB4 or EMT6 cells in 0.2ml saline. Tumor growth was monitored using calipers every 2 or 3 days. Tumor volume was calculated as (L\*W<sup>2</sup>)/2, (where L = length and W = width) for 4 to 5 weeks. After tumor size was measurable the mice, which have developed the cancer, were randomly divided into 2 groups (each one 6-8 mice). The control group was injected with saline, while the treated group was treated by IP injection of 100µl of different concentration of TCApFs (1×daily for the period of the experiment).

### Toxicology study

Toxicology study was done by Harlan Laboratories Israel, Ltd. (ISO 9001:2000 Certificate No.: US2002/3081). During the acclimation period, animals are randomly assigned to the various test groups, according to a table of random numbers. It is the principle of the test to assess the pharmacokinetics of the Test Item following a single intravenous (IV) injection to male and female Wistar Han<sup>TM</sup> rats, at three dose levels: 10×TED,

15×TED and 20×TED (TED – therapeutic effective dose). Two treatment cycles were done, interspaced by a 1-week interval, each confined to 1×daily repeated intravenous (IV) dosing sessions for 7 successive days. An additional group, injected with water for injection and DMSO solution under identical experimental conditions, serves as the Vehicle Control group. Blood is collected from jugular cannulas, which are surgically inserted approximately 48 hours prior to dosing, at 7 predetermined time points. The study is divided into a number of sessions, in order to reduce the number of animals to be bled simultaneously at each time point. All organs/tissues listed in the Study Protocol (under Appendix 2) were collected from the animals assigned to the toxicity phase during the respective scheduled necropsy and fixed in 10% neutral buffered formalin. Histopathological changes were described and scored by the Study Pathologist, using semi-quantitative grading of five grades: No Lesion, Minimal Change, Mild Change, Moderate Change and Marked Change.

#### Blood test

Samples of blood serum from patients with different type of cancer and blood serum of healthy persons were purchased from Asterand Partners in Human Tissue Research (SF 3807, USA). Samples were kept under -80°C. Amount of native TCApF in the blood was analyzed by standard Elisa procedure with TCApF polyclonal antibody.

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