Evaluation of anti-inflammatory and anti-viral activities of herbal drugs Anosohip and Votalek

F I N A L R E P O R T

In connection with the implementation of paragraph 2 of contract with "IMMITEK", Republic of Macedonia and the National Centre of Infectious and Parasitic Diseases (NCIPD), Bulgaria

For the Study of "Evaluation of cytotoxic, anti-inflammatory and anti-anti-tumor activities of two nutritional supplements: Anosohip and Votalek"

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Cancer is one of the major reasons for human death in the last decade following cardiovascular diseases, infectious diseases and ischemic heart diseases. Current therapies of cancer include chemotherapy and radiotherapy although they both have severe side effects. Each type of cancer requiresspecific treatment, whichmakesitis the need for developmentof highly specific therapeutic agents [1].

Various immunotherapeutic approaches have been used for the treatment of cancer. The NCI, Bacillus Calmette-Guérin vaccine for tuberculosis that contains attenuated mycobacteria has been used for the treatment of superficial bladder cancer. Immunotherapy based on its tumor specific and immune modulatory properties [2, 3]. BCG is widely administered in cancers of the lung, lung cancers and melanoma.

In the last years, a number of therapeutic antibodies have been approved for clinical treatment in cases of breast cancer (Trastuzumab), non-Hodgkin (Rituximab) and Hodgkin lymphoma (Brentuximabvedotin), colorectal cancer (Panitumumab, Cetuximab), chronic lymphocytic leukemia (Ibritinumomab), and acute myelogenousleukemia (Gemtuzumabozogamicin) [4]. Immunotherapy with cytokines is another option for cancer treatment, e.g. IL-2 metastatic melanoma and renal cell carcinoma and IFN-alpha for Kaposi Sarcoma and for breast cancer therapy [5].

Different cancer vaccines have been developed for prevention and treatment of malignant diseases but only Sipuleucel-T is approved for therapy of advanced prostate cancer in cancer treatment is ineffective [6]. Many of the developed vaccines include cancer-cellulartumor antigens chemically conjugated to carrier protein with or without other adjuvant [7-9].

Today natural products and their compounds derived from plants or microbes are of great interest for an anti cancer research. This huge variety of chemical structures provides different mechanisms of action and specific effects used for anti tumor therapy. A number of natural compounds are designed to repairs, prevent or destroy malignant cells [10]. Anosohip and Votalek are herbal drugs containing a mixture of plant extracts, such as: *Echinacea purpurea*, *Hedera helix*, *Thuja occidentalis*, *Viola tricolor*, *Arctium lappa*, *Rosa canina*, *Taraxacum officinale*, *Lamium album*, *Urtica dioica*, *Fumaria officinalis*, *Symphytum officinale*, *Petroselinum sativum*, *Avena sativa*, *Humulus lupulus*, *Matricaria chamomilla* (BHRPL-PR, trapezoviscose, potato license) [PRC] or transcription factor like PPAR [10]. Special attention is placed on marine-derived anti-angiogenesis products, which suppress and control the spread/formation of vascular systems, supporting tumor growth and invasion. The majority of these substances act via inhibition of enzymes or factors crucial for the growth of new vessels [11].

C.26 murine model of colorectal carcinoma is classical tumor model, developed in 1975 and determined as an undifferentiated Grade IV carcinoma [49]. C.26 cells display high metastatic potential and low tendency to metastasize mainly to the lungs. Inoculated in syngeneic Balb/c mice C.26 cells cause high mortality. This model is used more than 35 years for studying the process of carcinogenesis and the cancer therapy.

The aim of the present work was to develop an experimentalmurine model of colon carcinoma and to investigate the anti tumor activity of new generated therapy.

Results

Mice

Female 10 weeks old Balb/c mice were obtained from Harlan Farm, Blackthorn, UK. The animals were kept under specific pathogen free (SPF) conditions and the manipulations were approved by the Animal Care Commission at the Institute of Microbiology in accordance with the international regulations.

Antibodies

FITC (Fluorescein isothiocyanate) conjugated anti-mouse CD8 and CD335, PE (Phycoerythrin) conjugated anti-mouse ANP, and CD19 and APC (Allophycocyanin) conjugated anti-mouse CD4 and CD19 antibodies were purchased from eBioscience, Frankfurt, Germany and used for FACS (Fluorescence activated cell sorting) measurements.

Cell line

Murine colon carcinoma cell line C-26 (ICTUX/BFT (#CCC™ 26, 26060) was cultivated in complete RPMI (Roswell Park Memorial Institute) medium 1640 medium (Gibco, Gaithersburg, MD) containing 10% FCS (fetal calf serum), 4 mM L-glutamine, 50 μM 2- mercaptoethanol and antibiotics at 37 °C / 5% CO2. The confluent monolayer cells (90% viability) were harvested and used for in vivo tumor cell transplantation.

Tumor model establishment

For selection of proper C.26 murine colon carcinoma model 2 groups of mice were challenged with 5×10 cells per mouse. The animals were observed for 14 weeks. Palpable solid tumors were observed 2 weeks after C.26 challenge followed by 6% survival 3 month rather C.26 challenge, which is acceptable for the model sequence. A group of mice injected with PBS only was used as a control group.

Treatment schedule

A group of female Balb/c mice (5 mice per group) was inoculated with C.26 cells (5×10cells/mouse) and the mice were fed three times per day with test substances (TS group).The TS contains 0,015 mg Anosohip and 0,2μl Votalek per dose. Control groups of mice were challenged with C.26 cells without treatment or PBS only (Fig. 1, Table 1).

was monitored by measuring palpable solid tumors once a week via inoculation and the tumor volume was determined. Every two weeks the animals from all groups were bled and the sera were kept frozen at -70 °C. All experiments were duplicated.

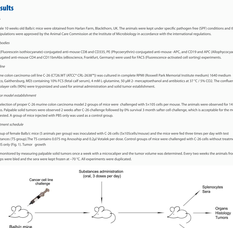

Figure 1. Treatment schedule

Phenotyping of spleen and tumor cell suspensions

We have performed an immune system modulation analysis during tumor development when the tumor model was established to demonstrate the immune reaction to the tumor growth. Organs from mice challenged with 5×10 C.26 carcinoma cells or PBS treated only were isolated andanalyzed by flow cytometry. Spleens and solid tumors from experimental animals (4 animals in + 4 to 6 were taken and mononuclear suspension was isolated by cell strainers (BD Bioscience, Erembodegem, Belgium). The cells were washed with PBS containing 2,5% FCS and 0,05% sodium azide and incubated with one of the following antibodies: anti-CD4-APC, anti-CD8-FITC, anti-CD335-PE, anti-CD19-PE-Cy5 and anti-CD11b-PE-Cy7 (eBioscience, Frankfurt, Germany) for 30 min at 4°C. Finally, the cells were washed twice and kept at 4°C. Ten thousand cells were analyzed from each sample with a BD LSR II flow cytometer using the Diva 6.1.1. software (BD Biosciences, San Jose, CA).

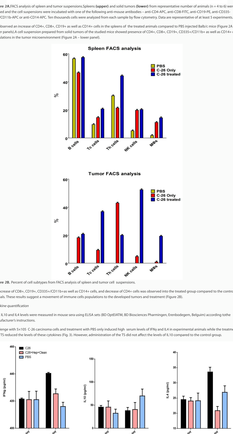

Figure 2A FACS analysis of spleen and tumor suspensions.Spleens (**upper**) and solid tumors (**lower**) from representative number of animals (n = 4 to 6) were excised and the cell suspensions were incubated with one of the following antibodies – anti-CD4-APC and CD8-FITC, anti-CD19-PE, anti-CD335-PE/CD11b-APC-or anti-CD19-APC. Ten thousands cells were analyzed from each sample by flow cytometry. Data are representative of at least 3 experiments.

We observed an increase of CD4+, CD8+ cells as well as of CD19+ cells in the spleens of the treated animals compared to PBS treated Balb/c mice (Figure 2A upper panel).A cell suspension prepared from solid tumors of the studied mice showed presence of CD4+, CD8+, CD19+, CD335+/CD11b+ as well as CD19+ cell populations in the tumor microenvironment (Figure 2A – lower panel).

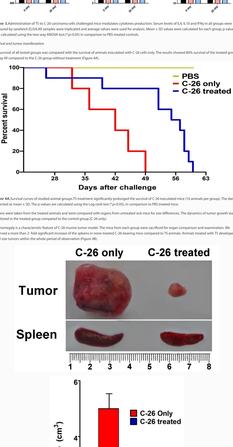

Figure 2B. Percent of cell subtypes from FACS analysis of spleen and tumor cell suspensions.

An increase of CD4+, CD19+, CD335+/CD11b+ as well as CD19+ cells, and decrease of CD8+ cells was observed into the treated group compared to the control animals. These results suggest a movement of immune cells populations to the developed tumors and treatment (Figure 2B).

Cytokine quantification

IFNγ, IL10 and IL6 levels were measured in mouse sera using ELISA sets (BD OptiEIATM, BD Biosciences Pharmingen, Erembodegem, Belgium) according tothe manufacturer's instructions.

Challenge with 5×10E C.26 carcinoma cells and treatment with PBS only induced high serum levels of IFNγ and IL6 in experimental animals while the treatment with TS reduced the levels of these cytokines (Fig. 3). However, administration of the TS did not affect the levels of IL10 compared to the control group.

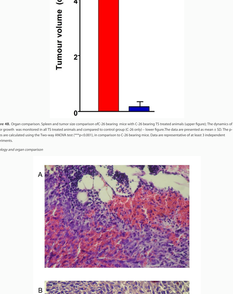

Figure 3 Administration of TS of C.26 carcinoma cells challenged mice modulates cytokines production. Serum levels of IL-6, IL10 and IFNγ in all groups were measured by sandwich ELISA.All samples were testpolicated and average values were used for analysis. Mean ± SD values were calculated for each group.p values were calculated using the two-way ANOVA test (*p<0,05) in comparison to PBS treated controls.

Survival and tumor manifestation

The survival of all tested group was compared with the survival of animals inoculated with C.26 cells only. The results showed 80% survival of the treated group on day 49 compared to the C.26 group without treatment (Figure 4A).

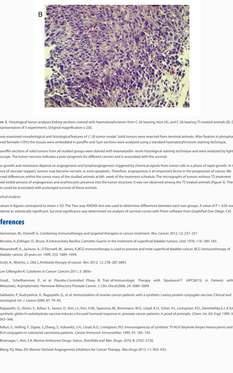

Figure 4A Survival curves of studied animal groups.TS treatment significantly prolonged the survival of C.26 inoculated mice (10 animals per group). The data are presented as mean ± SD. The values are calculated using the Log rank test (*p<0,05, in comparison to PBS treated mice).

Spleens were taken from the treated animals and were compared with organs from untreated sick mice for size differences. The dynamics of tumor growth was monitored in the treated group compared to the control group (C.26 only).

Splenomegaly is a characteristic feature of C.26 murine tumor model. The mice from each group were sacrificed for organ comparison and examination. We observed a more than 2- fold significant increase of the spleens in mice treated C.26-bearing mice compared to TS animals. Animals treated with TS developed small size tumors within the whole period of observation (Figure 4B).

Figure 4B. Organ comparison. Spleen and tumor size comparison of5-,C6-bearing mice with C-26 bearing TS treated animals (upper figure). The dynamics of tumor growth was monitored in all TS treated animals and compared to control group C.26 only) – lower figure.The data are presented as mean ± SD. The p values are calculated using the Two way ANOVA test (***p<0,001), in comparison to C.26 bearing mice. Data are representative of at least 3 independent experiments.

Histology and organ comparison

Figure 5. Histological tumor analysis.Kidney sections stained with haematoxylin/eosin from C.26 bearing mice (A), and C.26 bearing TS treated animals (B). Data are representative of 3 experiments. Original magnification x 250.

We have examined histological and histological features of C.26 tumor model. Solid tumors were excised from terminal animals. After fixation in phosphate buffered formalin (10%) the tissues were embedded in paraffin and 7μm sections were analyzed using a standard haematoxylin/eosin staining technique.

The paraffin sections of solid tumors from all studied group were stained with hematoxylin- eosin histological staining technique and were analyzed by light microscope. The tumor necrosis indicates a poor prognosis for different cancers and is associated with the survival.

Tumor growth and metastasis depend on angiogenesis and lymphangiogenesis mitigated by chemical signals from tumor cells in a phase of rapid growth. In the present work we investigated the effect of TS on tumor angiogenesis. Histological analysis of tumor sections from experimental animals (Figure 5) showed differences within the tumor mass of the studied animals at 60th week of the treatment schedule. The micrographs of tumors without TS treatment showed visible process of angiogenesis and erythrocytes presence into the tumor structure. It was not observed among the TS treated animals (Figure 5). These results could be associated with prolonged survival of these animals.

Statistical analysis

The values in figures correspond to mean ± SD. The Two-way ANOVA test was used to determine differences between each two groups. A value of P < 0.05 was considered as statistically significant. Survival significance was determined via analysis of survival curves with Prism software from GraphPad (San Diego, CA).

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