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Development of Small Molecules as Potential Therapeutics for Rheumatoid Arthritis

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MSc Molecular Biomedicine-Mechanisms of Disease, Molecular-Cellular Therapies and Bioinnovation

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Abstract

Chronic inflammatory diseases such as Rheumatoid Arthritis (RA), psoriasis and intestinal inflammation affect a total of 2-3% of the worldwide population. Regarding the diseases' etiology, a variety of factors have been documented to contribute, including genetic and environmental background of the individuals. The current therapies used in clinic include the Disease-Modifying Antirheumatic Drugs (DMARDs) and the Non-Steroid Anti-Inflammatory drugs (NSAIDs). In more severe cases, and after studies that proved the pathogenic role of TNF in these diseases, anti-TNF monoclonal antibodies are prescribed. Although biological agents are very efficient in remitting the disease, they are associated with several drawbacks, including high cost, inadequate clinical response, parenteral administration, as well as increased risk of several infections, due to the progressively diminished immune response. Therefore, there is a clear need for per os administered, well-tolerated, inexpensive drugs to treat RA and other diseases related to pathogenic TNF.

In this project we aim to identify new small molecules inhibitors for the treatment of chronic inflammatory diseases. To do so, we used two different approaches. The first was based on Synovial fibroblasts, a cell type that has been shown to underlie the pathogenic mechanisms of TNF dependent RA. The publicly available database L1000CDS^2 was used to identify small molecules that can reverse the diseased SFs signature. L1000CDS^2 proposed two kind of compounds a. an already approved antiphychotic drug and b. kinase inhibitors, targeting the kinases PLK-1 (polo-like kinase-1) and MnK-1/2. The second chemoinformatic structure based approach tried to identify small molecules that could serve as inhibitors of tpl-2 (tumor progressor locus-2) kinase as it has been shown that its activation promotes inflammatory response, in a macrophage dependent manner.

Introduction

Rheumatoid arthritis (RA) is the most common chronic inflammatory disease that is characterized by systemic manifestations and affects mainly the joints. RA' prevalence is 0.5-1 % in the general population, the disease onset occurs mostly in the age of 50 while it is more commonly met in women (80 %). The main clinical symptoms of the disease include joint inflammation (synovitis) and joint stiffness that occur due to synovial lining thickening and influx of inflammatory cells in the joint which in parallel with increased survival and proliferation of resident cells, lead to synovial hyperplasia, usually referred as pannus. RA is frequently accompanied by several comorbidities and long- term complications, such as osteoporosis, atherosclerotic lesions, myocardial infarction, stroke, insulin resistance and type 2 diabetes, depression, lymphomas and several infections (**1,2,3**).

Regarding the pathogenesis of RA, there is a combination of genetic variations and environmental factors that contribute to the initiation of the disease. More specifically, over the last years several genome wide association studies (GWAS) revealed numerous susceptibility genes, SNPs and genetic risk loci, predisposing to the development of RA. Furthermore, several posttranslational modifications, like citrullination and carbamylation of

proteins might contribute to this disease. Presentation of citrullinated peptides or other neo-epitopes from citrullinated proteins could activate autoreactive T cells, which in turn could induce B cell to help and stimulate the production of ACPA autoantibodies (**2**). Circulating ACPA enter the joint, bind to the citrullinated proteins, and form immune complexes (**4,5**). Increased production of proinflammatory cytokines, including TNF, IL-1, and IL-6, in turn recruit more immune cells into the joint, perpetuating the inflammatory process.

In addition, there has been increasing evidence, that another type of cells in the synovium has a major role in the initiation of RA (especially in patients, who are negative for ACPAs), named synovial fibroblasts (SFs). SFs are non-vascular, non-epithelial, CD45-negative cells of mesenchymal origin. Their physiological role is the production of nutrients for the cartilage and lubrication of the bone surfaces, while they assist to the influx of other cell types to the synovial space, by the adhesion of these cells to the extracellular matrix (**14**). RA SFs contribute to inflammation, angiogenesis, and matrix degradation of the joint by producing inflammatory cytokines and MMPs, resulting finally in joint destruction (**15**). Interestingly, it has been shown that pathogenic SFs can induce RA even in the absence of immune background, as isolated human RA SFs induced arthritis upon transfer to the knee of healthy SCID (immunodeficient) mice (**17**, **18**).

The first line treatments of RA often include corticosteroids (e.g., prednisone), Non-steroid anti-inflammatory drugs (NSAIDs) (e.g., aspirin, nimesulid, ibuprophen, indomethacin etc.) and disease-modifying antirheumatic drugs (DMARDs), such as the antimetabolites methotrexate and leflunomide which are dihydrofolate reductase inhibitors (previously used as anti-cancer drugs, mostly in lymphomas, by inducing immune cell death), chloroquine, D-penicillamine, gold sodium thiomalate and aurothioglucose (**6**). Since the above-mentioned drugs are lacking specificity, they are accompanied by many serious adverse reactions. Over the last years, in the framework of the development of more targeted therapies, it has been found that specific cytokines like TNF-a, IL-1 and IL-6 were involved in the pathogenesis of the disease.

TNF-a (Tumor Necrosis Factor-a) is a proinflammatory cytokine, produced in response to infection or immunological injury. It is activated by trimerization and it is found in 2 forms (transmembrane and soluble). TNF binds to two transmembrane receptors: TNFRI (receptor mainly for the soluble TNF) and TNFRII (receptor mainly for the transmembrane TNF) (**7**). It was found that TNF-a, plays a major role in RA pathogenesis, since transgenic mice (hTNFtg / tg197 mice), carrying a 3'-modified human TNF transgene (TNF-globin), show upregulated patterns of TNF expression and develop chronic inflammatory polyarthritis, a disease similar to human RA that is characterized by infiltration of inflammatory cells, synovial hyperplasia, cartilage destruction and bone erosion. Interestingly, treatment of these arthritic mice with a monoclonal antibody against human TNF, completely prevents the development of the disease (**8,9,10).** Those discoveries led to the development and approval of novel biological agents and biosimilars to treat RA patients, as well patients that suffer from other chronic inflammatory diseases,when the first line therapies are not effective. The anti-TNF biologics include anti-TNF-a monoclonal antibodies (Infliximab, adalimumab, certolizumab pegol and golimumab) and the recombinant soluble human TNF receptor Etanercept (**11,40**).Underlying the leading role of SFs in the RA context there was evidence that production of TNF in RA SFs is sufficient to initiate the disease, since, similarly with the human studies, hTNFtg / tg197 mice SFs injection to the knees of non-transgenic or of RAG

KO immunocompromised mice, induced high incidence of disease histologically (**16**). It has also been found that endogenous upregulated TNF or exogenous TNF administration leads to SFs hyperproliferation and that TNF/TNFRI signaling in SFs is sufficient and required for full development of RA and other TNF dependent diseases (**19, 20, 21**).

Other RA targeted therapies include monoclonal antibodies targeting IL-6 receptor (tocilizumab) or IL-6 itself (sirukumab, sarilumab) (**12**), the anti-CD20 monoclonal antibody rituximab, the anti CD80/86 CTLA-4 immunoglobulin abatacept (impaired autoantigen presentation), the IL-1 receptor antagonist anakinra and the anti-IL1-β monoclonal antibody canakinumab (**13**).

Despite the fact that all the above biologic therapies are characterized by increased specificity, they present several disadvantages, like loss of response over time, through the production of anti-drug antibodies, often serious side effects (fever, severe infections, leucopenia, neutropenia etc.), parenteral administration (intravenous/subcutaneous/ intraperitoneal) and high cost. As a result, there is a medical need for the development of new, more effective and safer therapies.

Furthermore, we studied the development of inhibitors of the tpl-2 kinase. Tpl-2 (tumor-progressor locus-2) is a Serine/Threonine kinase on chromosome 18, that is involved in various types of cancer and was first identified as a tumor promoter in virus induced T-cell lymphoma and breast carcinoma, as it activates MAP kinases, through a Ras and Raf dependent manner and in this way it promotes cancer drug resistance (**27,28,36**). Tpl-2 was initially isolated from a human carcinoma cell line while it was identified as a proto-oncogene that encodes for a serine threonine kinase of the MAP3K family , and thus is also known as MAP3K8 or Cot (Cancer Osaka Thyroid) (**29**). (**30**). However, Tpl-2 has also been shown to present tumor suppressing activities in colitis driven tumorigenesis, by acting in intestinal myofibroblasts (**31**).

Moreover, it has been published, that Tpl-2 kinase, apart from its original well characterized role as a tumor promoting gene, it is also involved in innate and adaptive immune responses. Tpl-2 becomes activated by an array of proinflammatory factors like IL-1, LPS, TNF-a, and in a stimulus and cell specific manner can affect the phosphorylation of MAP Kinases, p38, JNK, ERK and the activation of NFκB, and is critical for the production of inflammatory mediators like TNF-a, IL-1, Cox2, PGE2 and IL-6, in different models of inflammation (**32,33,34,35,37,38**). In addition it was shown that TNF-a production via an LPS stimulus, is regulated by an tpl-2/ERK dependent pathway, whereas it is linked with the NFkB pathway as well (**25,39**). The tpl-2 kinase is also linked with the promotion of autoimmunity, loss of immune tolerance and Th1 (T-helper 1 cells) mediated inflammation (**43,44**). Furthermore, tpl-2 kinase is involved in inflammatory responses, caused by DAMPs (Damage Associated Molecular Patterns), also known as sterile inflammation (**45**).

It was also shown that that tpl-2 kinase is a drugable molecular target, since genetic ablation and pharmacologic inhibition of this protein reduces, either innate immune responses, by primary human monocytes and synoviocytes (**46**,**47**), or adaptive immune responses by primary human $CD8^+$ cytotoxic T-cells (CTLs) (**48**). Furthermore, genetic and pharmacologic inhibition of this kinase, ameliorates inflammatory bowel disease (IBD) pathology, in a model of experimental colitis (**49**), whereas it can attenuate the phenotype of EAE (Experimental Autoimmune Encephalomyelitis), through an irradiation resistant non- hematopoietic cell type (**50**).

Conclusively, new inhibitors for the tpl-2 kinase could serve as potential novel drugs, for chronic inflammatory disorders.. There have been a lot of attempts to design potent inhibitors of Tpl-2. More specifically, in 2 papers, published in 2005 and 2007, the authors synthesized and tested the structure-activity relationship and the binding affinity of(1.7)-naphthyridine-3-carbonitrile compounds, to Tpl-2 (**52,53**). They observed that some of those inhibitors, reduced the TNF-a production in primary human monocytes. Additionally, quinoline-3-carbonitriles were tested as potential tpl-2 inhibitors (**54**). These compounds reduced the LPS mediated TNF-a production in primary human monocytes, as well as the LPS/D-Gal induced TNF-a release, in mice. Furthermore, after the crystal structure of tpl-2 kinase was revealed (**51**), several imidazoquinoline derivatives were evaluated as potential tpl-2 inhibitors in a structure based drug design approach (**55**). The most promising inhibitors were selected through a series of assays, such as through their binding affinity in Cot/ tpl-2 kinase assay (**52**), their effect on the LPS induced Phospho-ERK induction and TNF production in human peripheral blood monocytes (PBMCs), as well as their potential to reduce the uric acid induced IL-1β production in human PBMCs. The best inhibitors were also tested in vivo, by using an uric acid induced synovitis model in dogs (**56**), whereas in vivo pharmacokinetic evaluation was performed in rats and dogs. A recently published paper showed also that tpl-2 kinase blockade in mice with unaltered levels of the ABIN-2 protein (A20-binding inhibitor of NF-κB-2), did not lead to allergic airway inflammation, (**57**). Thus, the advantage of tpl-2 inhibitors as potential therapeutics for chronic inflammatory diseases, is that on top of the aforementioned advantages of the small molecules they are expected not to have unwanted hypersensitivity reaction comorbidities. Finally, the inhibition of tpl-2 kinase in endothelial cells, has been shown to attenuate the VEGF regulated angiogenesis (**60**), indicating that tpl-2 inhibitors can also disrupt pathologic angiogenesis, occuring during tumor growth, or in other diseases, such as diabetic retinopahy.

Based on the above mentioned scientific evidence, we also tested a series of compounds, as Tpl-2 potential inhibitors, derived from a virtual structure based screening. We aimed not only to confirm if those potential tpl-2 inhibitors affect angiogenesis, but also to determine their effect on blood brain barrier integrity, since unpublished data of our lab indicate the intrinsic role of TPl2 in blood brain barrier permeability.

Materials and Methods

Transgenic Mice Genotyping

All 8-week-old mice, that were used for the SFs isolation were genotyped, to determine if they are transgenic (tg197) or wild type (WT), under the following procedure: DNA was extracted from mice tails in the second week of age, under the sodium chloride/isopropanol/ethanol protocol. Then, by using those DNA samples, a PCR analysis followed by an agarose gel DNA electrophoresis, were used to detect whether the abovementioned mice carry the TNF-β globin gene construct (8). The experimental samples (3 biological replicates of tg197 and WT mice, respectively), were re-genotyped after the mice sacrifice using the same procedures as mentioned above for further confirmation. For the PCR analysis, we used the following primers:

Forward: H TNF Gl S: 5'-TACCCCCTCCTTCAGACACC-3',

Reverse: H TNF Gl A: 5'-GCCCTTCATAATATCCCCCA-3'.

PCR reaction was performed on a thermal cycler, using 29 cycles of 93oC for 1 min (denaturing), 58.5oC for 45 sec (annealing) and 72oC for 1 min (elongation) with a custom-made Taq polymerase.

Isolation of Synovial Fibroblasts

SFs were isolated from the joints of 8-week old wild type and tg197 arthritic mice, according to Armaka M. et al, 2009 (**22**). All experiments were performed in the animal facilities of Biomedical Sciences Research Center (BSRC) "Alexander Fleming" under specific pathogen–free conditions. All experiments were approved by the Institutional Committee of Protocol Evaluation in conjunction with the Veterinary Service Management of the Hellenic Republic Prefecture of Attika according to all current European and national legislation and performed in accordance with the guidance of the Institutional Animal Care and Use Committee of BSRC "Alexander Fleming".

Murine Synovial Fibroblasts culture

SFs were cultured as described in the previously mentioned isolation protocol (**22**). Briefly, cells were grown initially at 37°C, 5% CO2 in complete Dulbecco's modified Eagle's medium (DMEM) (Gibco/Invitrogen, Paisley, UK) supplemented with 10% fetal bovine serum (FBS), 1% L-Glutamine and 100 Units/ml of penicillin/streptomycin/amphotericin until the first passage. After the first passage, cells were cultivated in fresh DMEM supplemented with 10% FBS, 1% L-Glutamine, and 1% penicillin/streptomycin at 37°C in a humidified incubator with 5% CO2. The isolated cells were monitored daily and culture medium was changed every 3-4 days. Fibroblasts were selected by continuous culturing for at least 15 days and 3 passages.

Purity Analysis of Synovial Fibroblasts

A flow cytometric analysis of passage 3 SFs was performed to assess the purity of those cells in culture. Four cell markers CD90, ICAM-1, VCAM and CD45 were used for the purity analysis. In detail, purified anti-mouse monoclonal CD90 antibody (Alexa Fluor 488, clone: 30-H12, concentration: 0.5mg/ml and Alexa 647, clone: 30-H12, concentration: 0.5mg/ml), anti-mouse CD45 antibody (APC/Cy7, clone: 30-F11, concentration: 0.2mg/ml) and anti-mouse VCAM antibody (Alexa Fluor 647, clone: 429, concentration: 0.5mg/ml) obtained from BioLegend were used. Furthermore, purified phycoerythrin (PE) hamster anti-mouse CD54 (ICAM) antibody (concentration: 0.5mg/ml) was purchased from BD Pharmingen. The culture of SFs is considered pure, under the following relative percentages: Synovial fibroblasts ≥ 95%, macrophages ≤ 5% (The resulting cultures are >80-90% CD90.2 positive, >85% VCAM-1 positive and >80% ICAM-1-positive) (**22**).

Stimulations- Treatments in SFs

SFs isolated from WT or tg197 mice, were treated under the following agents:

Recombinant Human TNF-a: PeproTech, Catalogue number: 300-01A. The stock used was at 100μg/ml in 0.1% BSA and stored at -20^{o}C. Final concentration used was 10 ng/ml in 1XDMEM.

Infliximab: Remicade (Janssen Biologics B.V.). 100mg/ 20ml stored at 2-8 ^{o}C. Further dilution in 1XDMEM was used to achieve 1μg/ml final concentration.

Antipsychotic Drug. Capsules, containing 50 mg of the active substance were diluted in DMSO solvent at 25mM, to achieve 1mM final concentration in 1XDMEM.

Sandwich Elisa Assays for Chemokines Detection

We cultured the SFs, isolated from the tg197 and wild type (WT) mice in a 96 well plate ($2X10^4$ cells per well), using complete Dulbecco's modified Eagle's medium (DMEM) (Gibco/Invitrogen, Paisley, UK) supplemented with 10% fetal bovine serum (FBS), 1% L-Glutamine and 100 Units/ml of penicillin/streptomycin. After 1 day in culture, the cells were placed in starvation medium (DMEM, 0.5% FBS), in order to be synchronized in the same phase of the cell cycle. Then the cells were treated under the previously mentioned drugs with or without TNF for WT and tg197 cells, respectively. 48h later, the supernatants were collected. Sandwich elisa assays were performed under the following protocols provided by RnD Systems: Duo set ELISA Development System for Mouse CCL20 (Mip3α), Catalogue Number: DY760 and for Mouse CCL5 (RANTES), Catalogue Numbers: DY478. Furthermore, we determined the viability of the cells (synovial fibroblasts), attached to the 96 well plate and therefore the toxicity of the above-mentioned compounds, by conducting the crystal violet assay (**23**) (An additional wash with PBS + $MgCl_2$, $CaCl_2$ was initially conducted in comparison with the original protocol. The final staining elution was performed with 33% acetic acid instead of methanol.).

TNF-TNFRI Elisa Assay

TNFRI at 0.1 μg/ml in PBS was plated and incubated overnight at 4 ^{o}C. The next day 4 washes with PBS-Tween-20 (PBST) (0.05%) and an 1hr blocking with 1% BSA solution (in PBS), followed. The compound dilutions preincubated with 0.025 μg/ml recombinant human TNF diluted in PBS, were then added for 1h at RT. After 4 washes with PBST, a primary anti-human TNF antibody (kindly provided by Prof. W. A. Buurman, University of Maastricht) in 1/5000 dilution (host: rabbit) was added (incubation for 1hr in room temperature). A secondary anti-rabbit antibody, conjugated with HRP (peroxidase labeled anti-rabbit, dilution factor: 1/5000, host: goat, clone: IgG(H+L), Catalogue Number: PI-1000, storage at 4 ^{o}C, Vector Laboratories Inc.) was also added (incubation for 1hr in room temperature). Finally, the signal was developed by the TMB substrate kit (Catalogue Number: 34021, Thermo Fischer Scientific) and the reaction was stopped by adding 2 Molar of H_2SO_4 solution. The measurements were conducted in the spectrophotometer at 450 nm.

Western Blot Analysis

SFs were plated in 60mm culture plates and after 12 hours in starvation media, the cells (SFs treated with the above-mentioned agents for 30min) were washed with ice-cold PBS. RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% SDS and 1% NP-40) with protease and phosphatase inhibitors was added and the cellular components were removed from the plates by using a cell-scraper and collected to eppendorf tubes. Cells were incubated with RIPA buffer at 4^{o}C for 30min and a centrifugation in 12000 g for 10min at 4 ^{o}C followed. The

protein concentration in the supernatants was determined by the Bradford assay, using the Bio-Rad protein dye kit (Catalogue Number: 500-0006). The proteins were separated by 10% (w/v) SDS-PAGE gel electrophoresis and electroblotted to nitrocellulose membranes. The membranes were blocked for 1hr with 3% BSA (albumin bovine fraction V powder, Catalogue Number:A/1278/46, Fischer Scientific)in TBS-Tween 20 solution and incubated overnight with the following primary antibodies: IkB (35-41 KDa, host: rabbit dilution factor: 1/1000, Catalogue Number: SC371, Santa Cruz), phospho-ERK (42-44 KDa, host: rabbit, dilution factor: 1/400, Catalogue Number: SC7383, Santa Cruz), actin (host: goat, dilution factor: 1/1000, Catalogue Number: SC1615, Santa Cruz). The next day after 3 x 10 min washes with TBST, the membranes were incubated in room temperature for 1hr, with the following secondary antibodies: HRP conjugated anti-rabbit (peroxidase labeled, dilution factor: 1/2000, host: goat, clone: IgG(H+L), Catalogue Number: PI-1000, Vector Laboratories Inc.) and HRP conjugated anti-goat (peroxidase labeled, dilution factor: 1/2000, host: horse, clone: IgG(H+L), Catalogue Number: PI-2500, Vector Laboratories Inc.). Finally, the peroxidase substrate was added to the membranes (Luminata Crescendo Western HRP substrate kit, Catalogue number: WBLUR0500, Millipore) and a chemiluminescence detection system (Bio-Rad) was used according to the manufacturer's instructions to scan and quantitatively analyze the protein bands.

Gelatin Zymography Analysis

We used the zymography procedure, in order to detect the matrix metalloproteinase activity (MMP9 and MMP2 secretion) in the supernatants, collected from SF culture plates (after treatment with sulpiride), as described in the Elisa chemokine detection protocol. The zymography assay protocol that we used, was described in a paper, published by Armaka et al in 2008 (**19**). Briefly, 10 μg of total protein per sample was separated by 8 % SDS-PAGE gel electrophoresis, containing 1 mg/ml gelatin , at 4^{o}C for 2.5-4hrs in 100 volts. After that step 2 x 30 min washes of the gels, with 2,5% Triton X-100 solution (to remove SDS from the gel) followed and eventually, an incubation with MMP activation buffer (50 mM Tris-HCl pH: 7.5, 5 mM $CaCl_2$, 0.02% NaN_3, 1 μM $ZnCl_2$) for 20hrs, at 37 ^{o}C (shaking) was used. The gels were stained for 30 min in 0.5% Coomassie blue R250 (10% acetic acid, 45% methanol) and finally destained with 10 min washes in a solution of 40% methanol/10% acetic acid for 1hr. A chemiluminescence detection system (Bio-Rad) was used according to the manufacturer's instructions to scan and quantitatively analyze the protein bands.

L929 TNF-induced Cytotoxicity Assay

3×10^4 *cells*/*well of the* NCTC clone 929 [L cell, L-929, derivative of Strain L] (ATCC® CCL-1™) cells were seeded on a 96-well flat-bottomed plate and incubated overnight at 37 °C, 5% CO2. Next day, compounds at different concentrations were pre-incubated with 0.3ng/ml Recombinant Human TNF-α (Peprotech, 300-01A) and 2μg/ml Actinomycin D (ThermoFisher Scientific, A7592) for 30min at RT and then added in triplicates on the cells monolayer. Culturing medium alone (DMEM, high glucose, GlutamaxTM Supplement, 10566016), medium with Actinomycin D, as well medium with both TNF and Actinomycin D were used as controls. 18-22h after, dead cells were removed by washing the plate with 1xDPBS (ThermoFisher Scientific, 14040133). The plate was then incubated for 10min at RT in order to fix the live cells which then were stained for 5min with 0,5% crystal violet. After washing repeatedly and drying at RT the stain was solubilized using 33% acetic acid. The

quantification was performed spectrophotometrically at 570nm and the values were calculated using the following equation:

$$\frac{100 * [(OD570\ of\ sample) - (OD570\ of\ TNF\ and\ ActD)]}{(OD570\ of\ ActD) - (OD570\ of\ TNF\ and\ ActD)}$$

Cytotoxicity assay

L929 cells were seeded and grown overnight as described above. Next day, different concentrations of the compounds in 1xDMEM were added in the cells in triplicates. After 18-22h a crystal violet assay (**23**) was performed (see above) and the stained viable cells were measured using a non-treated sample as a control.

Cell Culture of RAW 264.7 Murine Macrophage Cell Line

The RAW 264.7 Murine Macrophage Cells (ATCC, were cultured at 37°C, 5% CO2 in complete Dulbecco's modified Eagle's medium (DMEM) Glutamax (Gibco, LOT 1976751 REF 61965-026) supplemented with 10% fetal bovine serum (FBS), 1% Sodium Pyruvate 100x (catalogue number: 11360-036, LOT 1955371) and 100 Units/ml of penicillin/streptomycin until passage 10-12.

Flow Cytometry Analysis for Tpl-2/Phospho-ERK Signaling Detection in RAW 264.7 Murine Macrophage Cell Line

The cells were cultured in 6 well culture plates in DMEM Glutamax (Gibco, LOT 1976751 REF 61965-026) complete medium (supplemented with 10% fetal bovine serum (FBS), 1% Sodium Pyruvate 100x and 100 Units/ml of penicillin/streptomycin), until they were attached to the wells (approximately 3 hours in 5% CO2, 37°C). Then the culture medium was changed to DMEM Glutamax starvation medium (Gibco, LOT 1976751 REF 61965-026) (supplemented only with 100 Units/ml of penicillin/streptomycin) and the cells were incubated for 24 hours, in order to be synchronized in the same cell cycle phase. The cells then were pre-incubated with the potential tpl-2 inhibitors and the commercial tpl-2 inhibitor (as positive control) for 45 minutes in 5% CO2, 37°C and then stimulated with LPS (catalogue number: L2630, Sigma Aldrich, from Escherichia Coli O111:B4, Storage: 2-8°C), in a concentration of 200 ng/ml, for 30 minutes in the same conditions. The samples then were transferred to 4°C to stop the LPS induced phospho-ERK signaling. The cells were fixed, using the IC fixation buffer (125 ml INVITROGEN catalogue number: 00-8222-49, storage at 2-8 °C) for 20 minutes in 4 °C and then permeabilized using ice cold methanol for another 30 minutes. In order to avoid the non specific binding of the antibody for the phosphor-ERK staining in the IgG FCγ receptors II/III of the macrophages, the samples were incubated for 20 minutes in 4°C with the CD16/32 purified anti-mouse blocking monoclonal antibody (BIOLEGEND size: 500 μg, volume: 1 ml, catalogue number: 101302, isotype: Rat IgG 2a,λ LOT B255480 CLONE: 93, storage at 2-8°C). This blocking antibody was diluted in cell permeabilization buffer (Volume: 100ml catalogue number: catalogue number: 00-8333-56, storage at 2-8 °C, LOT 1956536). The cells were stained with the anti- H/M phospho-ERK ½ (T202/Y204) antibody (APC, CLONE: MILAN8R REF 17-9109-42, storage at 2-8 °C, AFFYMETRIX INC, San Diego, USA, volume: 0.5 ml) for 60 minutes in 4°C (Dark). This antibody was also diluted in cell permeabilization buffer (Volume: 100ml catalogue number: catalogue number: 00-8333-56, storage at 2-8 °C, LOT 1956536), in order to achieve intracellular staining. Finally, the samples were kept in 4°C (Dark) for overnight in cold PBS 1X and the next day the flow cytometry analysis followed.

Human Umbilical Vein Endothelial Cells (HUVECs) Culture

The Human Umbilical Vein Endothelial Cells (HUVECs) (Gibco life science technologies ,Catalogue number: C-003-5C) were cultured in T-75 (75 cm^2) culture flasks in M-200 medium (Gibco life technologies, catalogue number: M-200-500), supplemented with the Large Vessel Endothelial Supplement (LVES) (50X) (Gibco life technologies, Catalogue number: A14608-01). The cells were kept in the culture flasks, until they reached 80% confluency and they were used until passage 6. The culturing, freezing and thawing procedures for HUVECs were followed according to the company's instructions. The cells, the growth medium and the supplement are included in the company's angiogenesis starter kit (gibco life technologies, catalogue number: A14609-01).

In Vitro Vascular Permeability Assay

(24 well-plate assay) (Millipore, catalogue number:ECM644, Storage: 2-8 °C). A 24 well plate with collagen precoated inserts (provided in vascular permeability assay kit, Millipore, catalogue number:ECM644), was used. The inserts were hydrated with the supplemented HUVEC growth medium, incubated for 15 min in room temperature HUVEC cells, were cultured until 80% confluency as previously described. The cells were then trypsinized and 100.000-200.000 cells were seeded per insert, while the rest of the well was sufficiently covered with supplemented HUVEC growth medium. The 24 well plate was incubated for 72 hours, in order for the endothelial cells to form a monolayer. After the 72 hours incubation the inserts were transferred to a new 24 well receiver plate and in each well a sufficient amount of supplemented HUVEC growth medium was added. After 1h treatment with 10μM of each potential inhibitor, the cells were treated with 100ng/ml Recombinant Human TNF-a for 24h (PeproTech, Catalogue number: 300-01A, Storage: -20 °C). The next day a FITC-Dextran permeability treatment in 1/40 dilution ,was used in each insert. The plate was incubated for 20 min in room temperature (Dark) and the medium of each well, outside of the inserts was transferred in a black 96-well opaque plate. Finally, a measurement in a fluorescence plate reader with filters appropriate for 485 nm and 535 nm excitation and emission, respectively, was performed. The highest the abundance of the fluorophore in the medium, the lowest the integrity of the endothelial cell monolayer. Finally, the inserts were stained for 20 min in room temperature with a specific cell stain solution, provided in the assay kit, in order to detect the quality of the HUVEC monolayer in each insert microscopic (brightfield) imaging.

Results

In this project we aimed to identify small molecules that could serve as treatments for chronic inflammatory diseases. To do so, we performed a bioinformatic analysis, where the deregulated genes of tg197 derived SFs, before and after treatment with two anti-TNF monoclonal antibodies infliximab (Remicade) and adalimumab (Humira), were compared with gene signatures, produced by several pertubations on several cell lines. We used the Lincs1000 public repository (http://amp.pharm.mssm.edu/l1000fwd/main) and the L1000CDS2 tool (http://amp.pharm.mssm.edu/L1000CDS2/#/index), which is an ultra-fast publicly available gene signature search engine, that enables users to find compounds that, either mimic or reverse the user's input signature. L1000CDS² searches in a set of ~ 35000 different signatures produced by 62 human cell lines when treated with approximately 4000 compounds across a range of concentrations and time points (26). This tool proposed a. an already approved anti-psychotic drug and b. some kinase inhibitors, targeting the kinases PLK-1 (polo-like kinase-1) and MnK-1/2 kinases. Those inhibitors were previously developed as treatments for solid tumors and different types of leukemia, such as AML (Acute Myeloid Leukemia), since the PLK-1 and MnK1/2 kinases play a role in cell cycle **(41,42).** Furthermore, PLK-1 is a kinase, that was previously found upregulated in SFs, isolated from RA patients, compared to SFs derived from osteoarthritis (OA) patients **(58).**

To further test the proposed compounds, 8-week old tg197 and wild type (WT) mice SFs were cultured and treated with the anti-TNF monoclonal antibody infliximab and with a neuroleptic drug. The genotype of the mice, used in this experiment, was confirmed with PCR analysis (**Figure 1**).

a. An antipsychotic drug affects TNF pathway in Synovial Fibroblasts

a1. The Neuroleptic Drug presents an its Anti-inflammatory Effect on Synovial Fibroblasts by Reducing the Production of CCL5 and CCL20

To obtain evidence that this antipsychotic drug possesses anti-inflammatory properties in the arthritic SFs, we evaluated the production of the chemokines CCL5 and CCL20 from SFs, treated with this agent. CCL5, which is also called as RANTES (regulated on activation, normally expressed and secreted from T cells), is a chemotactic chemokine for T-cells, eosinophils and basophils and plays a role in recruiting leukocytes into inflammatory sites, while CCL20, which is known as MIP-3a (macrophage inflammatory protein-3) is a small chemokine, that is a chemoattractant for lymphocytes and neutrophils. CCL20 secretion can be induced by microbial proteins such as LPS or inflammatory cytokines like TNF-a and IFN-γ. After culturing and starving the cells for 24hrs, the cells were treated with the anti-TNF monoclonal antibody infliximab or with the neuroleptic drug for 48hrs in 37 ^{o}C. Moreover, the SFs, isolated from the joints of the WT mice were stimulated with exogenous TNF, to induce inflammatory response. The supernatants of those cells were used to detect the levels of the chemokines CCL-5 and CCL-20, by performing an ELISA assay. We also checked for the viability of the SFs and therefore for the toxicity of the neuroleptic drug, through the Crystal Violet assay. We observed a significant reduction of the increased levels of CCL5 and CCL20 upon treatment with the antipsychotic drug in non toxic concentrations. On the other hand we did not observe a reduction to the chemokines produced of the WT SFs, when they are treated with exogenous TNF-a, indicating an effect of the antipsychotic drug

to the effects of endogenous hTNF only (**Figure 2**). The benzamide neuroleptic was diluted in DMSO, thus relevant quantity of DMSO alone was added to the cells as a control, as this solvent can cause toxicity or anti-inflammatory responses (**24**). Infliximab was used as a positive control.

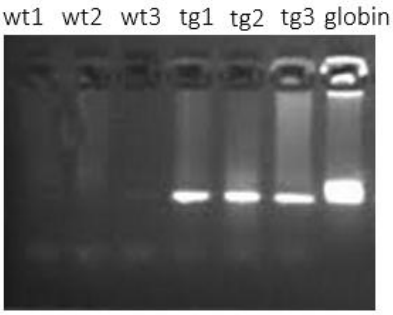


Figure 1: PCR analysis to detect the presence of the TNF-globin gene construct, in DNA samples isolated from the tails of 3 experimental biological replicates of 8weeks old WT and Tg197 mice.

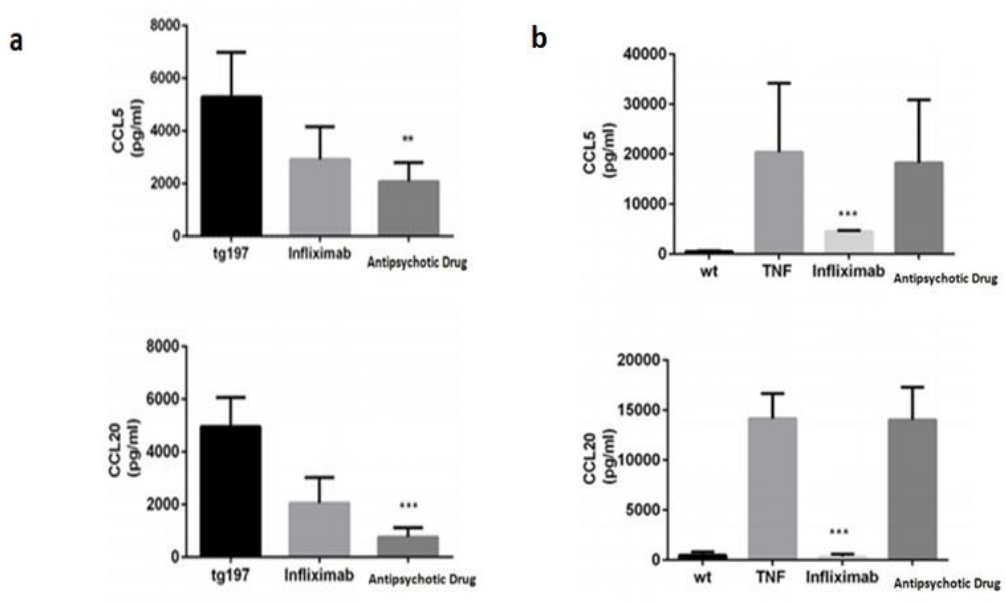


Figure 2: ELISA assay analysis for the detection of CCL5 and CCL20 chemokines levels in cell supernatants, collected from (**a**) tg197 arthritic mice synovial fibroblasts, upon treatment with the anti-TNF monoclonal antibody infliximab (1μg/ml in H_2O) or the antipsychotic drug (1mM) and (**b**) from WT mice SFs, treated with exogenous recombinant human TNF-a (10 ng/ml in 1X DMEM) alone or with hTNF + antipsychotic drug (1mM in DMSO) or infliximab (1μg/ml in H2O). The neuroleptic drug, was diluted in DMSO, so the reduction of CCL5 and CCL20 due to DMSO, was removed as background signal from the chemokines levels presented in the graphs.

a2. The antiphychotic drug exerts its anti-inflammatory action in Tg197 SFs through affecting ERK phosphorylation.

Tg197 SFs that have continuous endogenous upregulated TNF levels, were used for a protein extraction procedure and a western blot analysis. The western blot analysis revealed that treatment with the antipsychotic drug for 30min, as well as with infliximab for the same time caused a similar reduction in the TNF-a induced phospho-ERK activation. We also checked the NFkB pathway, but IκB degradation did not seem to change upon treatment with the neuroleptic drug, in SFs (**Figure 3**).

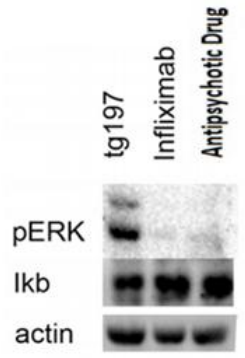


Figure 3: Western blot analysis of protein extracts, either isolated from tg197 arthritic mice synovial fibroblasts, upon 30min treatment with the anti-TNF monoclonal antibody infliximab (1μg/ml) and the antipsychotic drug (1mM).

a3. The Antipsychotic Drug is not a Direct TNF-a Antagonist

For further checking that the antipsychotic drug does not act as a direct TNF inhibitor, as it has no effect on the chemokines levels of the exogenously stimulated with TNF WT SFs, we conducted an Elisa type assay, in order to confirm if the addition of several concentrations of the neuroleptic drug, is sufficient to disrupt the binding of TNF-a to its main receptor TNFRI. Despite the fact, that this assay is fully *in vitro* and does not fully represent a cellular physiological model, the addition of increasing concentrations of the antipsychotic drug, did not reduce the TNF-TNFRI binding affinity, proving that this drug is not a direct TNF antagonist (**Figure 4**).

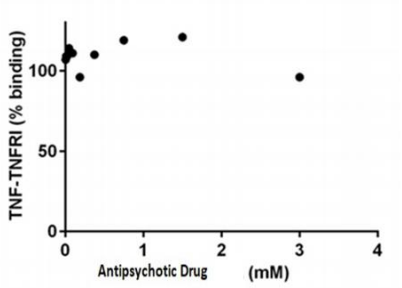
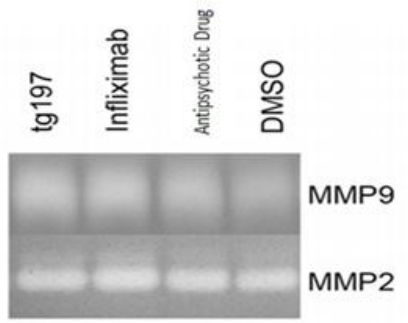


Figure 4: ELISA type assay analysis, regarding the TNF-TNFRI binding affinity, under the addition of increasing concentrations of the antipsychotic drug, diluted in DMSO.

a4. The Neuroleptic Drug, does not affect the expression of Matrix Metalloproteinases in the Synovium.

In order to test the antipsychotic drug's effect in the production of the matrix metalloproteinase MMP9, which is one of the protein factors responsible for the bone erosion in the joints of arthritic mice, we isolated SFs from joints of 8-week old WT and tg197 mice and after 24h starvation we treated them with the anti-TNF monoclonal antibody infliximab or with the neuroleptic drug with or without previous exogenous administration of TNF, respectively. After 48h treatment we collected the supernatants and we performed gelatin zymography, that revealed, that treatment with the antipschotic drug, does not affect the MMP9 production from synovial fibroblasts derived either from tg197 arthritic mice or from WT type mice when treated with exogenous human TNF-a, as similar slight attenuation of MMP9 was observed in the cells treated with the control solvent DMSO (**Figure 5, Figure 6**).

a.



b.

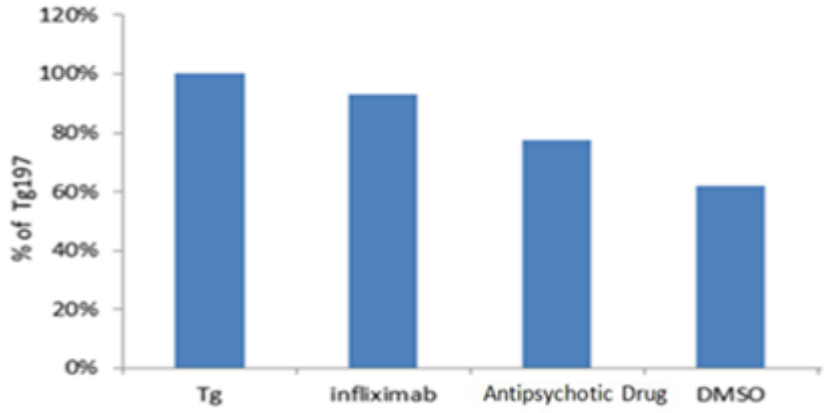
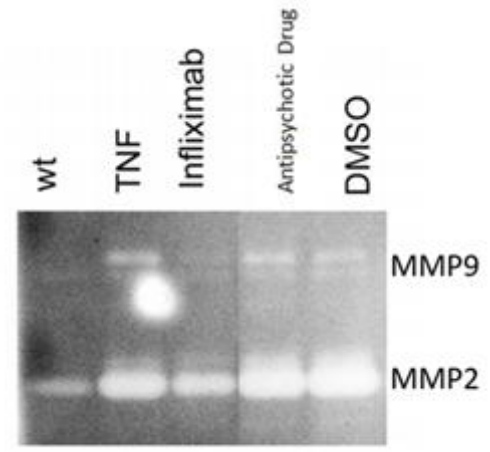


Figure 5: Gelatin zymography analysis of MMP9 protein levels in cell supernatants, collected from (**a**)tg197 arthritic SFs, upon treatment with the anti-TNF monoclonal antibody infliximab (1μg/ml in H_2O) or the antipsychotic drug (1mM in DMSO) for 48hrs. (**b**)MMP9 was quantified using MMP2 as a loading control. The neuroleptic drug, was diluted in DMSO which was used alone as a control.

a.



b.

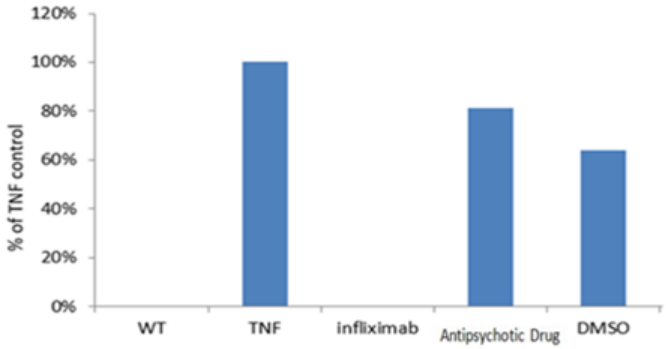


Figure 6: (**a**) Gelatin zymography analysis of MMP9 protein levels in cell supernatants, collected from WT mice SFs, treated with exogenous recombinant human TNF-a at 10 ng/ml alone or along with the antipsychotic drug (at 1mM in DMSO) / anti-TNF monoclonal antibody infliximab (1μg/ml in H_2O) for 48hrs (**b**). Quantification of MMP9 protein bands was calculated as to MMP2 quantity. The antipsychotic drug, was diluted in DMSO which was used as a control.

b. Effect of MnK-1/2 and PLK-1 Inhibitors in SFs

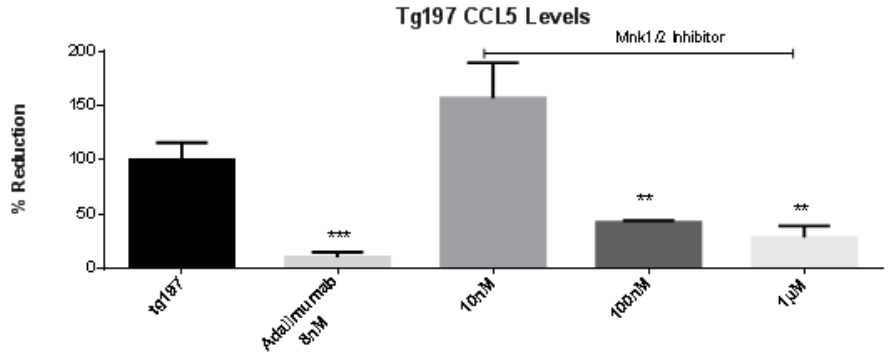
b1. MnK-1/2 and PLK-1 Inhibitors present an effect on CCL5 (Rantes) and CCL20 (Mip-3a) Production in Tg197 and WT Murine Synovial Fibroblasts upon exogenous hTNF stimulus

To obtain evidence that the proposed kinase inhibitors possess anti-inflammatory properties in the arthritic mice, we evaluated the effect of these compounds on the production of the chemokines CCL5 and CCL20 from SFs isolated from the joints of tg197 arthritic mice and of WT mice. After culturing and starving the cells for 24hrs, the cells were treated with the anti-TNF monoclonal antibodies infliximab (Remicade) and adalimumab (Humira) as positive controls, or with the MnK1/2 and PLK-1 kinase inhibitors for 48hrs in 37 ^{o}C. The cells supernatants were then collected and used to detect the levels of the chemokines CCL5 and CCL20, by performing an ELISA assay. The MnK1/2 inhibitor, was found to reduce the production of CCL5 and CCL20, in tg197 SFs supernatants, compared to the untreated

samples in concentrations of 100 nM and 1 μM (**figure 7**). Furthermore we evaluated the first PLK-1 inhibitor (Inhibitor A), in regards of its effect on the levels of the chemokines CCL5 and CCL20, in cell supernatants, collected from tg197 SFs. CCL5 and CCL20 levels were reduced, when SFs were treated with the inhibitor A, especially in concentrations of 100 nM and 1 μM, compared to the untreated samples (**figure 8**). We used as positive control the anti-TNF monoclonal antibody Humira (adalimumab) in a concentration of 8 nM (**figure 7** and **8**). However, inhibitor A also targets PLK2 and PLK3, so we ordered 2 more specific PLK-1 kinase inhibitors, inhibitor B and inhibitor C.

We treated the tg197 derived SFs with the inhibitor B in concentrations of 1,100 nM and 1μM for 48hrs in 37 ^{o}C. The levels of the chemokine CCL5 were reduced, compared to the untreated samples only in the concentration of 1μM (**figure 9a**), whereas the levels of the chemokine CCL20 were reduced in the concentrations of 100 nM and 1μM (**figure 9b**). As far as the inhibitor C is concerned, the tg197 derived SFs were treated in concentrations of 1, 10 and 100 nM, as well as, 1 and 10 μM. The levels of both the chemokines CCL5 and CCL20, were reduced, compared to the untreated samples only in the concentration of 10 μM (**figure 9a** and **b**). The anti-TNF monoclonal antibody Remicade (infliximab), was used as positive control in a concentration of 8 nM (**figure 9**).

a.



b.

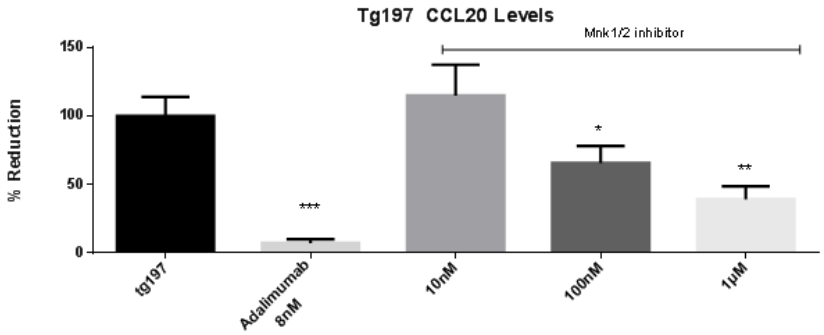
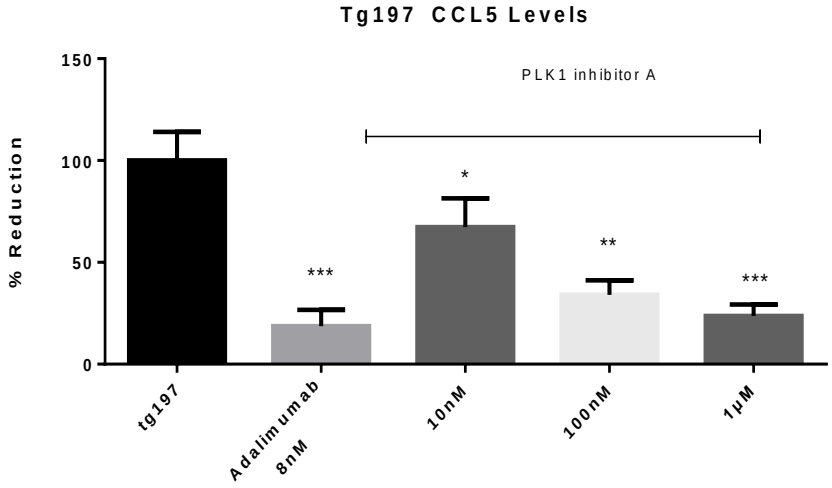


Figure 7: Levels of the Proinflammatory chemokines ccl5 (**a**) and ccl20 (**b**) in Tg197 SFs supernatants, after treatment with a MnK 1/2 inhibitor (10,100 nM and 1μM). Humira (adalimumab), was used as positive control in a concentration of 8 nM. Humira was diluted in H_20, whereas the MnK1/2 inhibitor was diluted in DMSO.

a.



b.

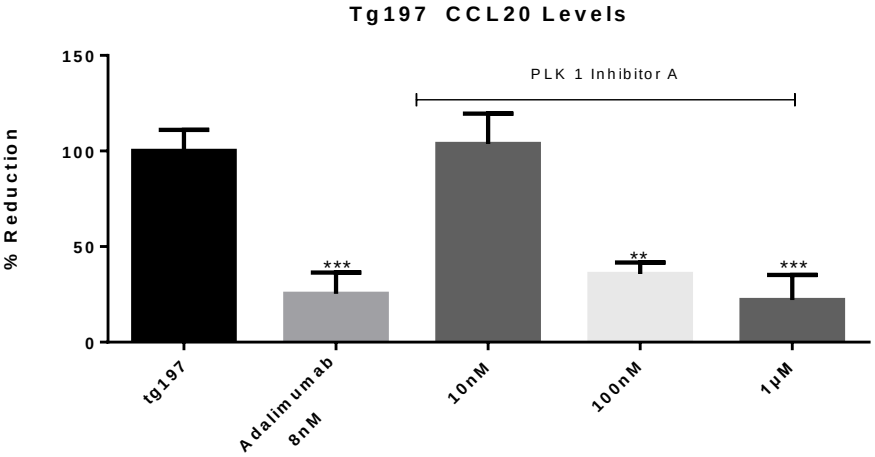
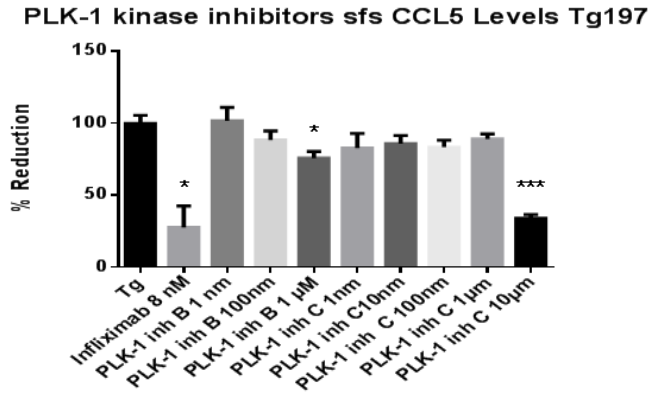


Figure 8: Levels of the Proinflammatory chemokines ccl5 (**a**) and ccl20 (**b**) in Tg197 SFs supernatants, after treatment of SFs with the PLK-1 inhibitor A (10,100 nM and 1µM). Humira (adalimumab), was used as positive control in a concentration of 8 nM. Humira was diluted in H_20, whereas the PLK-1 inhibitor A was diluted in DMSO.

a.



b.

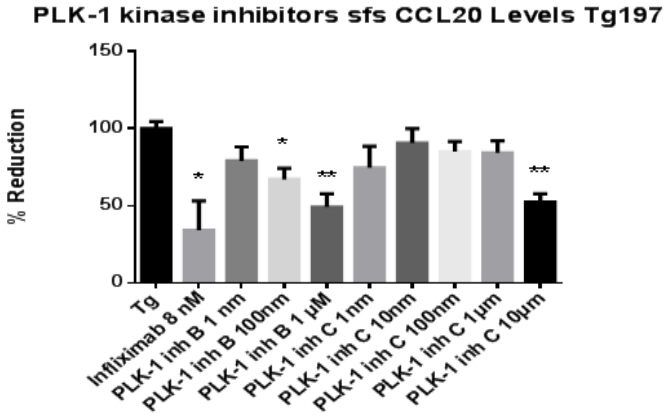
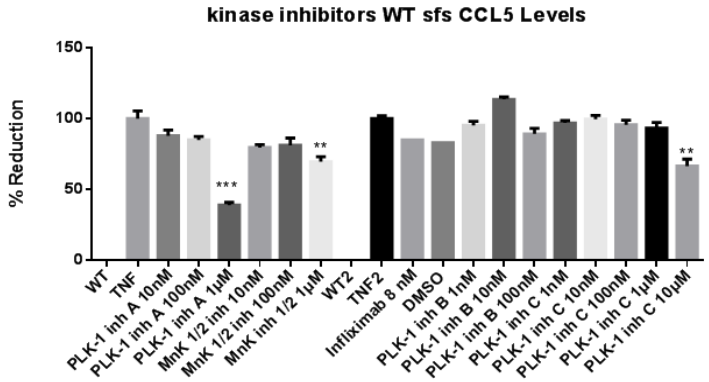


Figure 9: Levels of the Proinflammatory chemokines ccl5 (**a**) and ccl20 (**b**) in SFs cell supernatants, after treatment of SFs with the PLK-1 inhibitor B (1,100 nM and 1µM) and PLK-1 inhibitor C (1,10,100nM and 1,10µM). The SFs were isolated from the joints of tg197 arthritic mice. Infliximab, was used as positive control in a concentration of 8 nM. Remicade was diluted in H_20, whereas the PLK-1 inhibitor B and C were diluted in DMSO.

Furthermore the abovementioned PLK-1 and MnK1/2 inhibitors were evaluated in SFs, isolated from the joints of WT mice. After culturing and starving the cells for 24hrs, the cells were treated with the anti-TNF monoclonal antibody infliximab (Remicade) in a concentration of 8 nM as positive control, or with the MnK1/2 and PLK-1 kinase inhibitors for 48hrs in 37 ^{o}C. Moreover, the WT mice SFs were stimulated with exogenous TNF, to induce inflammatory response. MnK 1/2 inhibitor was used in concentrations of 10,100 nM and 1 µM. The levels of CCL20 were reduced in the WT SFs cell supernatants, in all three concentrations, compared to the sample that was treated only with exogenous TNF, whereas the levels of CCL5 were not altered (**Figure 10**). As far as the PLK-1 inhibitors are concerned, the WT SFs were treated with inhibitor A in concentrations of 10, 100 nM and 1 µM, with inhibitor B in concentrations of 1, 10 and 100 nM, as well as, with inhibitor C in concentrations of 1,10,100 nM and 1,10 µM. The levels of CCL20 were reduced in the WT SFs cell supernatants, by treatment with inhibitor A in all three concentrations, with inhibitor C only in the concentration of 10 µM, whereas remained unaltered when the WT SFs were treated with inhibitor B (**Figure 10**). The comparison was performed with the samples, that

were treated only with exogenous TNF. In addition the levels of the proinflammatory chemokine ccl5, were ameliorated in the WT SFs cell supernatants, only when the SFs were treated with with inhibitor A in a concentration of 1 μM and with inhibitor C in a concentration of 10 μM compared to the samples treated only with exogenous TNF (**Figure 10**), indicating that Compound A is the most effective as to inhibit the proinflammatory chemokines in WT SFs upon exogenous TNF stimulation.

a.



b.

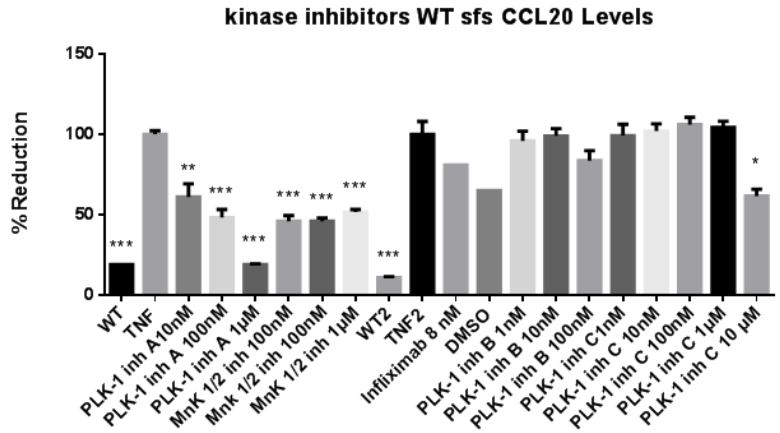
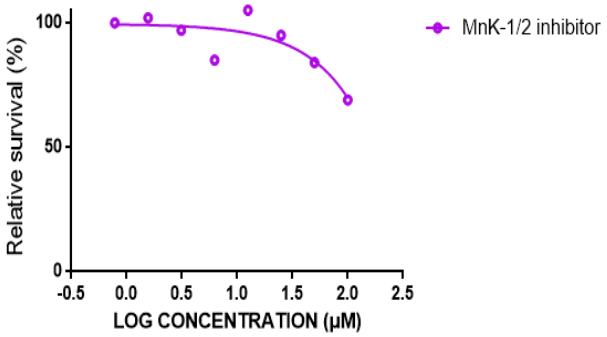


Figure 10: Levels of the Proinflammatory chemokines ccl5 (**a**) and ccl20 (**b**) in SFs cell supernatants, after treatment of SFs with the PLK-1 inhibitor A (10,100 nM and 1 μM), the PLK-1 inhibitor B (1, 10,100 nM) and the PLK-1 inhibitor C (1,10,100nM and 1,10μM). The SFs were also treated with the MnK-1/2 inhibitor (10,100 nM and 1 μM). The SFs were isolated from the joints of WT mice and treated with exogenous recombinant human TNF-a (10 ng/ml in 1X DMEM). Remicade (infliximab), was used as positive control in a concentration of 8 nM. Remicade was diluted in H_20, whereas the PLK-1 inhibitors and the MnK-1/2 inhibitor, were diluted in DMSO.

B2. Evaluation of the MnK-1/2 and PLK-1 Inhibitors' Toxic Effects

We checked for the SFs cell viability under treatment with the PLK-1 inhibitors A,B and C, as well as, with the MnK1/2 inhibitor in various concentrations, through a crystal violet toxicity assay (**23**). It was found that the PLK-1 inhibitors were toxic for SFs in concentrations greater than 5μM, thus the reduction in the levels of the chemokines CCL5 and CCL20, in concentrations greater than 5μM, may occur due to the attenuated cell viability. Furthermore, the MnK-1/2 inhibitor was toxic in concentrations greater than 50μM (**Figure 11**). As the kinase inhibitors seemed to affect TNF pathway we tested them in another TNF dependent assay, studying their potential for the inhibition of TNF-a induced cytotoxicity in L929 fibrosarcoma cell line (**59**) (see Materials and Methods for details). We observed, that among the PLK-1 and MnK-1/2 inhibitors, the PLK-1 inhibitor C, inhibited most effectively the Actinomycin D induced toxic effects in the L929 cells, whereas the most succesful (in SFs) Mnk-1/2 inhibitor and PLK1 inhibitor A managed to affect the L929 TNF induced death, but in higher concentrations (**Figure 12**).

a.



b.

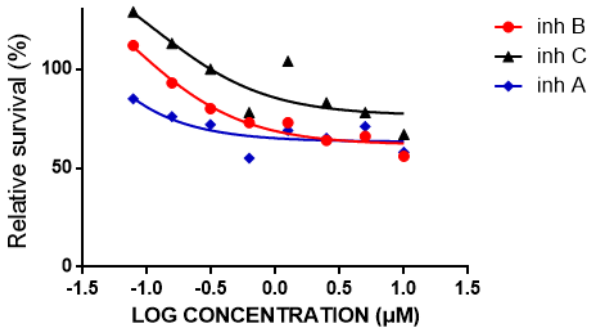
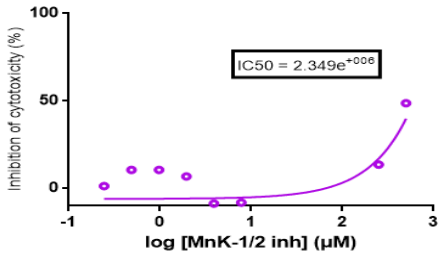
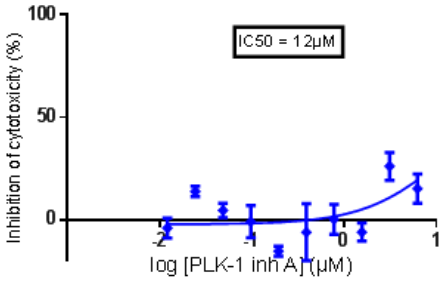


Figure 11: Relative Survival of SFs under treatment with the MnK-1/2 inhibitor (**a**), as well as the PLK-1 inhibitors A,B and C (**b**) in various concentrations. The viability of the cells, was measured in 96-well plates, by the crystal violet toxicity assay. The MnK-1/2 inhibitor was toxic in concentrations greater than 50μM (**a**), whereas the PLK-1 inhibitors A,B and C, appeared to be toxic in concentrations greater than 5μM (**b**).

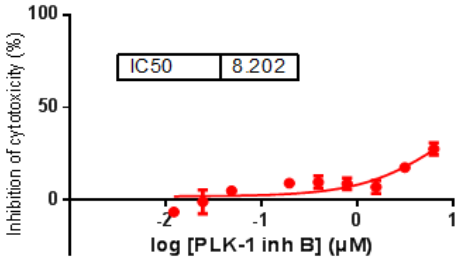
a.



b.



c.



d.

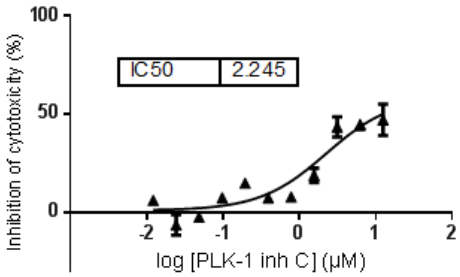


Figure 12: Inhibition of TNF-a induced cytotoxicity in L929 fibrosarcoma cell lines by the MnK-1/2 inhibitor (**a.**) and the PLK-1 inhibitors A (**b.**) B(**c.**), C(**d.**) The cells are treated with the inhibitors in various concentrations for 18-22hrs. The compounds were pre-incubated with 0.3ng/ml Recombinant Human TNF-α and 2µg/ml Actinomycin D for 30min at RT before treatment.

B3. The MnK-1/2 and PLK-1 Inhibitors are not Direct TNF-a Antagonists

The addition of increasing concentrations of these compounds did not reduce the TNF-TNFRI binding affinity, proving that they are not direct TNF antagonists (**Figure 13**).

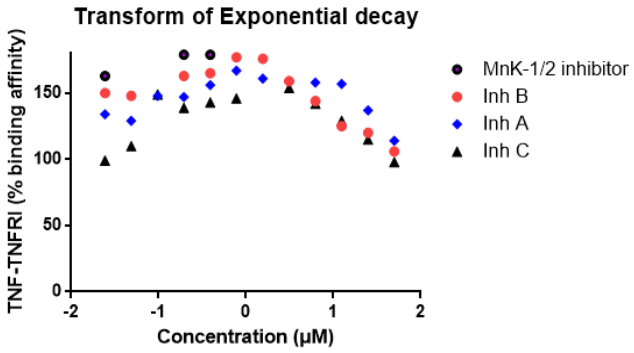


Figure 13: ELISA type assay analysis, regarding the TNF-TNFRI binding affinity, upon the addition of increasing concentrations of the PLK-1 inhibitors (A.B and C) and the MnK-1/2 inhibitor, diluted in DMSO.

Taking into account the above mentioned results MnK-1/2 inhibitor and PLK-1 inhibitor A are the best compounds, since they reduced the CCL5 and CCL20 chemokine production significantly in non toxic concentrations. Although PLK-1 inhibitor C, demonstrated the best inhibition of the TNF induced cytotoxicity in the L929 assay, it reduced the CCL5 and CCL20 chemokine production significantly in toxic concentrations, as the crystal violet toxicity assays indicated.

C. Tpl-2 kinase inhibitors

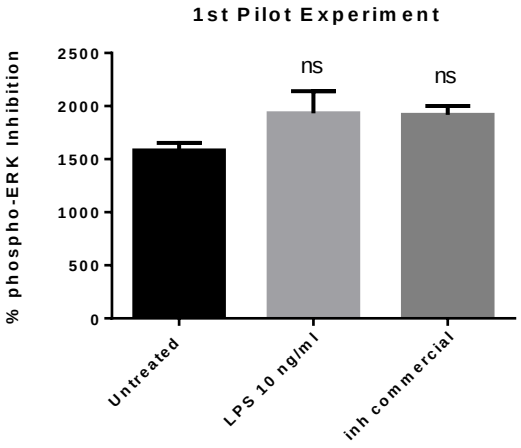
Following a chemoinformatic approach and based on the 3D structure of Tpl2 kinase we identified commercially available compounds that could potentially bind to the kinase and inhibit its function.

C1. Evaluation of Potential Tpl-2 Kinase Inhibitors in RAW 264.7 Macrophages Cell Line

We tested the efficacy of 20 compounds tested as potential tpl-2 inhibitors. Those compounds demonstrated the best score in an in silico virtual screening, regarding the binding affinity in the tpl-2 kinase. The compounds were evaluated for their efficacy to attenuate the LPS induced phospho-ERK activation in RAW 264.7 macrophages cell line. In order to determine the appropriate quantity of the LPS treatment for the phospho-ERK induction, we performed 2 pilot experiments, where the cells were treated with 4 different concentrations of LPS (10, 100, 200 ng/ml and 1 μg/ml) and with a commercial tpl-2 inhibitor in a concentration of 10 μM. The results indicated, that the most appropriate concentration of LPS for the phospho-ERK activation in the RAW macrophages cell line is above 200 ng/ml (**Figure 14**). Thus, the cells were pre-treated with these compounds, as well as with a

commercial tpl-2 inhibitor as a positive control for 45 minutes in a concentration of 10 μM and then stimulated for 30 minutes with LPS in a concentration of 200 ng/ml. A flow cytometry analysis followed with intracellular staining with an anti-phospho-ERK antibody, in order to detect the phospho-ERK induction in the samples treated with LPS and the above mentioned inhibitors. As a result, we observed, that 7 out of the 20 compounds that were tested, reduced significantly the LPS induced phosphor-ERK signaling, indicated that are potential tpl-2 inhibitors (**Figure 15**).

a.



b.

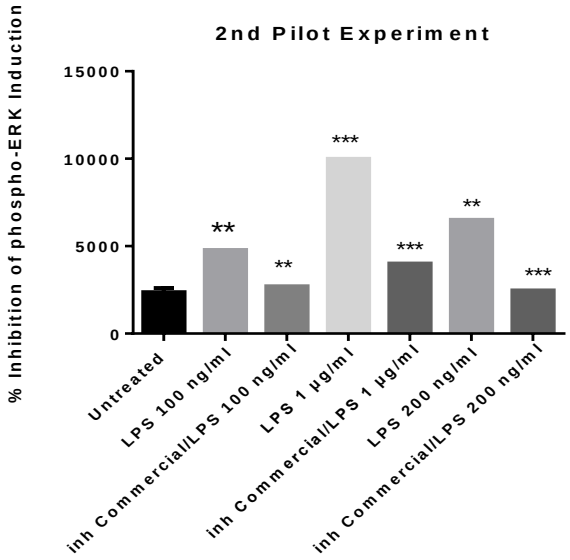
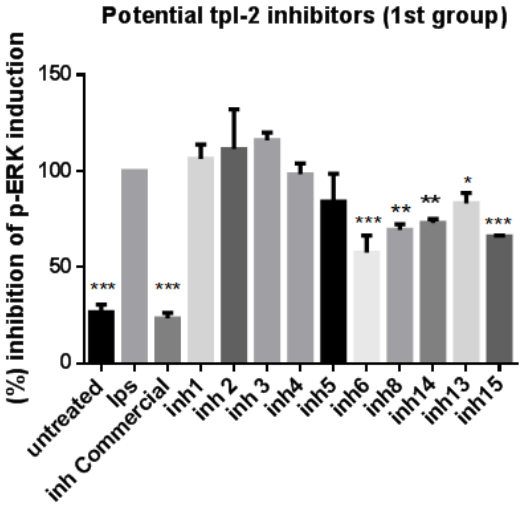


Figure 14: Flow cytometry analysis pilot experiments for the determination of the most appropriate LPS concentration for the phospho-ERK induction in RAW macrophages cell line. The commercial tpl-2 kinase inhibitor was used in a concentration of 10 μM in both experiments. LPS was used in a concentration of 10 ng/ml, with no significant phospho-ERK activation (**a**), whereas significant phospho-ERK induction was achieved, when used in a concentration of 100, 200 ng/ml and 1 μg/ml (**b**). Based on the above mentioned results we selected to proceed with the concentration of 200 ng/ml for the evaluation of the potential tpl-2 kinase inhibitors.

a.



b.

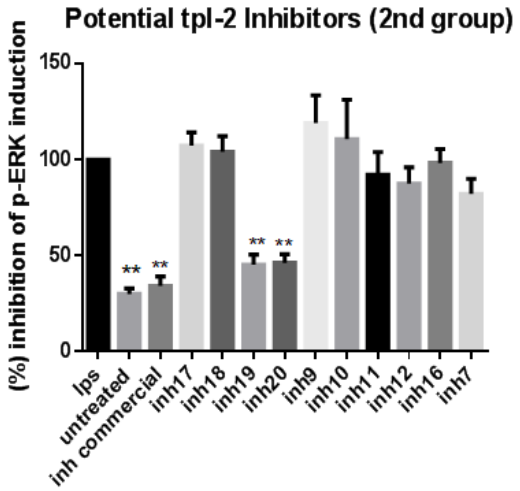


Figure 15: Flow Cytometry analysis for the detection of inhibition of tpl-2/ phospho-ERK signaling pathway in RAW macrophage cell line, after treatment with potential inhibitors in a concentration of 10 μM. Induction of inflammation and activation of the tpl-2/ERK pathway, was produced after treatment with LPS (200 ng/ml). The compounds tested, as well as, the commercial tpl-2 inhibitor (Positive Control), were diluted in DMSO.

C2. Assessing the Effect of the Most Successful Inhibitors in Blood Brain Barrier Integrity

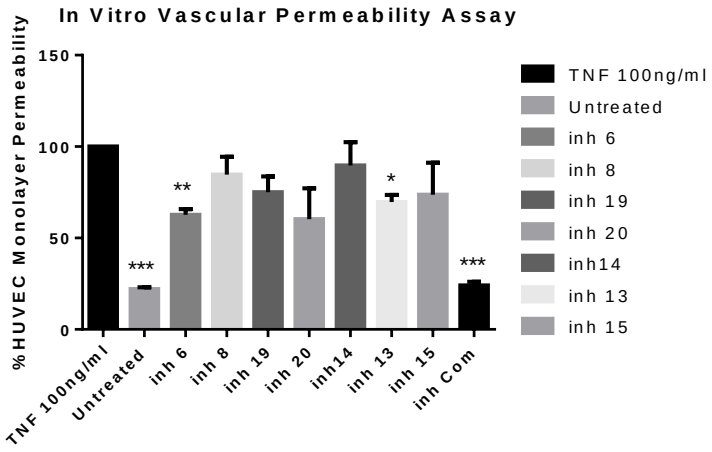
The most promising compounds were tested on human umbilical vein endothelial cells (**HUVECs**), in order to determine their effect in the cells' integrity, through a Transwell permeability assay.

Our goal is to determine whether the most successful potential tpl-2 inhibitors (selected by the abovementioned flow cytometry analysis screening), improve the Blood Brain Barrier integrity, upon inflammatory conditions, like the ones that occur in chronic inflammatory diseases eg. in Multiple Sclerosis (MS). To do so we used an in vitro assay which resembles the Blood Brain Barrier integrity, using HUVECs, similar to other assays published in the

scientific literature (**61**). The monolayer of HUVEC cells cultured in collagen precoated inserts, was pretereated with the most successful potential tpl-2 inhibitors at a concentration of 10 μM. A 24-hour treatment with Recombinant Human TNF-a which was used as a vascular permeability factor followed. A multitude of vasoactive cytokines, growth factors, and signal modulators react with endothelial cell substructural components to control permeability. Vascular endothelial growth factor (VEGF), interleukin-1 alpha and beta (IL-1α and IL-1β), tumor necrosis factor-alpha (TNF-α), and interferon gamma (IFN-γ) have been shown to increase endothelial monolayer permeability (**62,63,64,65**). We then performed a FITC-Dextran permeability treatment, in each insert. The greater amount of the FITC-Dextran that could be transferred, through the HUVEC monolayer in the insert, to the rest of the well, the higher the permeability of the endothelial cell monolayer was.

As a result, out of the seven potential tpl-2 kinase inhibitors selected from the abovementioned flow cytometry screening, two seemed to reduce the increased permeability of the monolayer, after the treatment with the vascular permeability factor (Recombinant Human TNF-a). Furthermore, we observed, even greater rescue of the monolayer permeability, under the treatment with the commercial tpl-2 inhibitor, which was used as a positive control (**Figure 16 a**). The cell monolayer was confirmed upon cell stain using microscopic (brighfield) imaging (**Figure 16 b**).

a.



b.

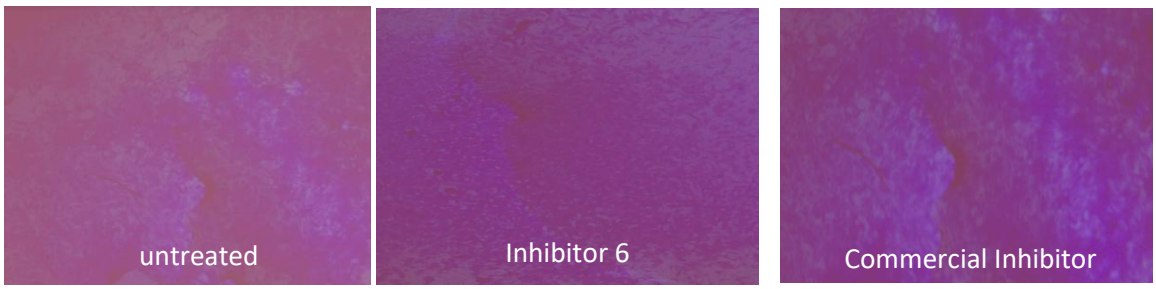


Figure 16: (**a.**) In Vitro Vascular Permeability assay to determine whether the 7 most successful in the abovementioned flow cytometry screening, potential tpl-2 inhibitors, could rescue the HUVEC monolayer

integrity, after treatment with recombinant human TNF-a, as a vascular permeability factor in a concentration of 100 ng/ml and incubation in 37 ^{o}C for 24 hours. Before the TNF-a treatment the HUVEC monolayer was pretreated with the potential tpl-2 inhibitors, in a concentration of 10 μM and incubated at 37 ^{o}C for 1 hour. We observed a significant reduction of the vascular permeability with the commercial tpl-2 inhibitor (used as positive control), as well as, with the potential tpl-2 inhibitor 6 and 13.(**b.**) Example of microscopic brightfield imaging of the HUVEC monolayer in the inserts, after the cell staining.

Discussion

According to the previously mentioned results from the ELISA type assays, the western blot analysis and the gelatin zymography experiments, the benzamide neuroleptic drug, possess anti-inflammatory properties, regarding the synovium of tg197 arthritic mice, since it ameliorates the production of the proinflammatory cytokines CCL5 (RANTES) and CCL20 (MIP-3a) from SFs, isolated from the joints of the arthritic mice, while, it did not affect significantly the production of the matrix metalloproteinase MMP-9, which might be responsible for the bone erosion in the joints. Thus, it is expected that the antipsychotic drug is mainly acting in the inflammation part of the arthritis. Furthermore, treatment of arthritic synovial fibroblasts with this antipsychotic drug, caused a significant reduction in the phospho-ERK activation, but not in the Ikb degradation indicating that the anti-inflammatory action of the drug is not mediated through the Nfkb pathway. Our results also confirmed that the neuroleptic drug does not act as a direct anti-TNF-a antagonist, as expected since the drug has no effect on WT SFs upon exogenous TNFa stimulation.

From the panel of kinase inhibitors that were tested, the Mnk1 and Plk1 inhibitor A (which were the most successful) were also found to interrupt the production of the pro-inflammatory chemokines without interrupting TNF-TNFRI binding.

The above inhibitors should also be tested in acute and chronic inflammation models to test their effect *in vivo*. For an acute model the sepsis like LPS induced mouse model could be used, with the chemokines levels in mouse serum to be the readout. Concerning the chronic inflammation model, as we started from the Tg197 SFs, the arthritic transgenic Tg197 mouse is the most appropriate to be used. The pathology of this model is accessed by an extrernal arthritis scoring as well by a histological analysis concerning the influx of the inflammatory cells (synovitis), the bone erosion and the cartilage destruction.

To further test the molecular pathways that are implicated in the function of the antipsychotic drug, as well as of the most successful MnK-1/2 and pLK-1 inhibitors on SFs , an additional approach would be to set up a high throughput phospho-proteomics experiment, by preparing samples for a mass spectrometry analysis. The phoshoproteomics experiment, through pathway analysis, will also reveal off- target binding that is maybe contributing to the anti-infammatory potential of the antipsychotic drug and the MnK-1/2 and PLK-1 inhibitors. A medicinal chemistry approach could then be used to modify the pharmacophore structure of the compounds, in order to create new small molecules, not covered by patents, with improved efficiency against chronic inflammatory diseases . Finally, in vivo testing of the new derivatives in the tg197 mouse model, as well as in vitro cell-based assays of those small molecules in SFs isolated from biopsies in the synovium of RA patients, are appropriate experiments to further evaluate their action against chronic inflammatory diseases.

Regarding the potential tpl-2 inhibitors project, in order to determine more accurately the effect of the tpl-2 inhibitors in the blood brain barrier integrity, we could test them on blood brain barrier organoids, which simulate better the blood brain barrier function and structure (**66**). Furthermore, the most promising compounds, could be tested for their selectivity, by evaluating their binding affinity, towards tpl-2 kinase in comparison with other kinases, directly involved in the tpl-2 pathway upstream of phospho-ERK. In parallel, dose response experiments, regarding the tpl-2 kinase binding, should also be performed. The inhibitors with the best IC50 values could then be optimized, through organic synthesis approaches, to create novel, more effective compounds. The optimized compounds should then be tested in vivo, by using the LPS-D-galactosamine mouse model, which resembles acute inflammatory conditions and the Experimental Autoimmune Encephalomyelitis mouse model which corresponds to Multiple Sclerosis conditions. Finally, another virtual screening, through chemoinformatics approaches could be performed, in order to follow a repurposing approach, by determining the most promising potential tpl-2 inhibitors, among FDA approved drugs. These drugs would then be tested in the above mentioned drug development pipeline.

Conflict of Interest

The authors state no conflict of interest

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