THE ROLE OF CAPSAICIN-SENSITIVE SENSORY NERVE ENDINGS AND SEMICARBAZIDE-SENSITIVE AMINE OXIDASE IN MOUSE TUMOR MODELS

Doctoral (Ph.D) thesis

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INTRODUCTION

1. Cancer as a therapeutic challenge

Cancer is a significant public health challenge worldwide, including in Hungary. The increase in incidence and mortality rates, as well as the diverse biological behaviour of tumours, justify an intensification of research. Osteosarcoma (OS), breast cancer and melanoma are tumours with different immunogenicity, vascularity and microenvironmental characteristics, which deserve special attention due to the difficulty of cure and high mortality rates (1). Osteosarcoma (OS) is a primary bone cancer affecting young adults and adolescents, whose treatment is based on a combination of surgery and chemotherapy. The biology of the tumour, its genetic instability, and chemoresistance require new targeted therapies that act at the molecular level. It most commonly occurs near the metaphysis of the long bones, particularly at the distal femur and the proximal end of the tibia. The tumour is derived from mesenchymal stem cells (MSCs) that develop in the direction of osteogenic differentiation and is thought to arise from osteoblast precursors at some stage of MSCs' preosteoblast-directed differentiation (2). Breast cancer is the most common malignancy in women, and its heterogeneity (hormone receptor status, HER2 status) requires complex treatment strategies. Breast tumours usually originate from the excessive proliferation of ductal cells, with the tumour microenvironment, including stromal cell effects or macrophages, playing a key role in their progression (3). Several factors, including tumour type, stage, hormone receptor status, HER2 status, general health status and patient preferences, determine the treatment of breast cancer. Previous research has shown that capsaicin-sensitive afferent nerve fibres have antitumour and immune-system effects, although the exact mechanism of these effects is not yet fully understood. Several studies have demonstrated the antitumour effects of capsaicin, which are attributed to its apoptosis-promoting, cell cycle-inhibiting, and vascularisation-reducing (anti-angiogenic) properties, among others (4-6). Given the firm reliance of TNBC-type tumours on new blood vessel formation, the orthotopic mouse model we have created using the 4T1 cell line is an excellent model for studying tumour growth and its microvascular characteristics. Melanoma, one of the most aggressive forms of skin tumours, is a key area for the development of immunotherapies and molecularly targeted treatments due to its immunogenic properties. Melanocytes are pigment-producing cells located in the basal layer of the skin that deliver melanin-filled melanosomes to neighbouring keratinocytes, protecting against the harmful effects of ultraviolet (UV) radiation. Melanoma can evade immune surveillance by secreting

inflammatory and immunosuppressive molecules, expressing inhibitory receptors, and releasing tolerogenic cytokines, thereby creating an immune-avoidant microenvironment. In addition, it induces angiogenesis to overcome oxygen and nutrient starvation, thereby supporting tumour growth. This multi-step, complex process is a significant challenge for both researchers and clinicians, as it complicates the accurate diagnosis and treatment of melanoma (7). Melanoma is one of the most immunosensitive tumours, and immunotherapy, in particular checkpoint inhibitory antibodies, is therefore a key part of its treatment. The efficacy of therapy is primarily influenced by the infiltration of effector cells into the tumour tissue, for which the expression of endothelial cell adhesion molecules is essential. Targeted therapies, such as BRAF inhibitors and viral-based treatments, as well as new developments like mRNA vaccines, represent a promising direction based on a deeper understanding of the disease (8).

2. Modelling opportunities in cancer

Understanding and researching OS requires the development of new modelling systems that provide opportunities to study it in more detail and to test new therapeutic approaches in preclinical trials. The first OS cell line to be established and still frequently used is the K7M2 cell line, which we use, derived from spontaneously developing lung metastatic OS in BALB/c mice (9). Depending on the research objective, other rodent OS cell lines exist, such as LM8 and the Dunn cell line (10). Different animal models, such as zebrafish models, are also increasingly used. In addition, several human OS cell lines have been successfully generated, including MG63, SaOS and 143B (9). Breast cancer is a highly heterogeneous disease, and even within a single tumour, there is considerable heterogeneity. The most widely used model animals are mice and rats; however, non-mammalian animals, such as zebra finches, can also be used. Xenograft models are particularly prevalent as they can be used to study the behaviour of human tumours in vivo. The 4T1 mouse cells we use were cultured from breast tumour cells derived from BALB/c mice and have spontaneous metastatic potential when implanted as allografts. The disease progression of this animal model is clinically similar to that of advanced malignant human breast cancer (11,12), and the 4T1 breast cancer metastasis model has been widely used in preclinical research (13,14). A better understanding of melanoma development and progression requires the use of optimized tumour models of different complexity. The simplest and most commonly used experimental models are 2D cell cultures (e.g., monocultures of melanoma cell lines), which enable the rapid investigation of various experimental conditions; however, they do not accurately reflect the complexity of the in vivo environment. The most commonly used animal models are zebrafish and mouse models, which are widely

used to study melanoma in vivo and have provided considerable insight into the biology of this complex tumour type. Several different research mouse models of melanoma have been developed, including our melanoma allograft model, B16, which is derived from a spontaneously developing melanoma tumour isolated from a C57BL/6 mouse strain (15, 16).

3. Role of transient receptor potential vanilloid 1 (TRPV1) and ankyrin 1 (TRPA1) receptors and capsaicin-sensitive nerve terminals

Among the best-known members of the transient receptor potential (TRP) family of receptors directly cation channel-coupled receptors are TRPV1 and TRPA1, which are functionally expressed not only on the cell bodies and peripheral terminals of primary sensory neurons (e.g., in dorsal root and trigeminal ganglia) but also on a variety of non-neuronal cell types (17,18). The TRPV1 ion channel is a member of the TRP family, which plays a crucial role in sensing various physical and chemical stimuli and is particularly important in regulating nociceptive sensation, inflammatory processes, and several pathophysiological mechanisms. Structurally, TRPV1 is a protein of 838 amino acids with six transmembrane domains (S1-S6) and an ion-permeable pore located in a loop formed between the S5 and S6 segments. The ionic conductance of the channel allows the transfer of mainly calcium and sodium ions, which activate intracellular signalling pathways (19). The most crucial exogenous activator of TRPV1 is capsaicin, the active substance in hot peppers. TRPV1 is primarily expressed in primary sensory neurons, but is also present in non-neuronal cells, including keratinocytes (20,21), mast cells (22), lymphocytes (23), and chondrocytes or cells of the gastrointestinal system (24). A variety of exogenous and endogenous agonists, as well as physicochemical stimuli, regulate TRPV1 function. The most prominent exogenous activators are capsaicin, resiniferatoxin (RTX), and allicin, which bind to the receptor's "vanilloid pocket" (located between the S3-S4 and S5-T550 regions). Capsaicin not only activates but also desensitizes the receptor (25). TRPV1 can also be activated by various animal toxins (e.g. scorpion and spider venoms). Endogenous activators include endocannabinoids (e.g., anandamide, N-arachidonyldopamine), derivatives of arachidonic acid, as well as protons (pH < 6) and the painful heat stimulus (>43 °C). Prostaglandins (26) and bradykinin (27) also play a role in sensitization. Non-selective inhibitors include ruthenium red and capazepine (28). Selective inhibition of the receptor may play a crucial role in pain relief, particularly in inflammatory and neuropathic pain, as demonstrated by SB366191 (29) and AMG 517 (30). Another effective antagonist is JNJ-17203212, which reduces pain sensitivity in both in vitro and in vivo models, while eliciting similar thermoregulatory side effects (31). TRPA1 has a similar structure to the TRPV1

receptor. Among the TRP receptors identified in mammals to date, TRPA1 has the most significant number of ankyrin repeat domains in its N-terminal region, comprising 17 ankyrin repeat domains. This ion channel has a broad activation spectrum, which can be activated by a variety of exogenous (e.g., cinnamaldehyde, allyl isothiocyanate, and low temperatures <17°C) and endogenous irritants or inflammatory mediators (e.g., formalin, hydrogen peroxide)(32) .Endogenous activators include 4-hydroxy-2-nonenal (33), hydrogen sulfide (34), 15-delta-prostaglandin J2 (35), and semicarbazide-sensitive amine oxidase (SSAO) products such as formaldehyde (36), hydrogen peroxide (37), and methylglyoxal. Several antagonist compounds (HC-030031, SV-1287) are involved in the regulation of TRPA1 channel function (38,39).

4. Capsaicin-sensitive sensory nerve endings

Since the 1970s, our research group has been studying a specific group of thin myelin sheath (Aδ) and non-myelinated (C) sensory fibres, the so-called capsaicin-sensitive sensory nerve endings, and the ionotropic TRP receptors located on them. Nociceptors expressing TRPV1 and TRPA1 receptors form a special subset of peripheral afferent nerve fibres. These so-called peptidergic, capsaicin-sensitive sensory nerve terminals account for about 40-50% of Aδ- and C-fibres. Their specificity lies in their unique triple role: in addition to their afferent function, they can exert both local and systemic efferent effects. Recently, increasing evidence suggests that these channels are involved not only in tumour pain reduction but also in tumour formation and progression. Over the past decade, several studies have demonstrated that various ion channels, including TRP channels, are expressed in human tumours and may play a role in several cellular processes, such as proliferation, migration and invasion (40,41).

5. Expression and role of TRPA1 and TRPV1 channels in cancer

Functional expression of the TRPV1 receptor has been demonstrated in several tumour types, including human breast cancer cell lines (MCF-7, BT-20), papillary thyroid carcinoma (BCPAP), prostate adenocarcinoma (LNCaP, PC-3) (42), urothelial carcinoma (43), and glioma cells (44). In preclinical mouse models, TRPV1 antagonists such as capsazepine or resiniferatoxin reduced tumour-induced pain, suggesting a possible role for TRPV1 in the development of tumour-associated hyperalgesia. Furthermore, TRPV1 expression has been found in femoral innervation sensory neurons, and inhibition of the receptor or repression of its gene has reduced pain responses in mouse models (45). In conclusion, the TRPV1 channel plays a critical role in both tumour cell proliferation and tumour-associated pain. It is a promising target for the development of antitumor and analgesic therapies. TRPA1 expression has been

shown in several tumour types. Our group has previously successfully detected TRPA1 and TRPV1 expression in the K7M2 osteosarcoma cell line using the RNA scope in situ hybridization technique (46). Our results suggest that TRPA1 expression may also be involved in the development of breast cancer pain, for example, by its detected presence in breast tumour tissues. This indicates that the TRPA1 receptor is also a promising target for the development of new analgesic drugs, particularly in conditions such as pain associated with bone tumours.

6. Semi-carbazide-sensitive amine oxidase (SSAO) and its role in tumourigenesis-related processes

SSAO, also known as vascular adhesion protein-1 (VAP-1), is a member of the copper-containing amine oxidase enzyme family and is responsible for the oxidative deamination of primary amines in the body. This biochemical reaction produces aldehyde, hydrogen peroxide and ammonia as end products (47). Over the last 15 years, intensive research has focused on the development of various small-molecule SSAO inhibitors for therapeutic purposes; however, their clinical application has been limited by several factors. Some inhibitors lacked selectivity because they also inhibited other amine oxidases, such as MAO, while others had inadequate physicochemical properties, including poor solubility or toxic structural features (48). Two main types of small-molecule SSAO inhibitors are known: irreversible and reversible inhibitors (49). Several selective, irreversible inhibitors have been extensively studied, including hydrazine, allylamine, and oxime-type compounds. LJP-1207 (N'(2-phenyl-allyl)-hydrazine hydrochloride) and BTT-2052 (1S,2S-2-(1-methylhydrazinyl)-2,3-dihydro-1H-inden-1-ol) are effective in various rodent inflammation models but are not suitable for clinical development due to their toxicity (49,50).

7. New multi-target SSAO inhibitor compound: SzV-1287

A new SSAO inhibitor compound with a complex mechanism of action, SzV-1287 (3-(4,5-diphenyl-1,3-2-yl) propanal oxime), patented by our group, is an oxime analogue of the known cyclooxygenase (COX) inhibitor oxaprozin (51). SzV-1287 is an innovative, metabolism-activated, multi-target compound that acts as an active prodrug. Its chemical structure has been designed to be converted into a COX-inhibitory active metabolite, oxaprozin, under predominantly acidic conditions (such as inflammation and hypoxia). At the same time, the parent molecule also possesses irreversible SSAO inhibitory activity. Our research team has also demonstrated that SzV-1287 not only inhibits the SSAO enzyme but also exerts a direct inhibitory effect on the TRPA1 and TRPV1 ion channels located on sensory nerve cells (39).

SV-1287 is a unique so-called metabolism-activated multi-target drug (MAMUT) (51). This novel drug development approach has been demonstrated to be effective in several animal models of inflammation and pain. The anti-inflammatory effect of SV-1287 has been demonstrated in both acute (carrageenan-induced) and chronic CFA inflammation models (52-54). The drug development process of SzV-1287 commenced with preclinical studies from 2016 to 2020, yielding positive results. Currently, the human phase IA (single ascending dosing) has been completed (Patent No. WO/2015/159/112, WO/2010/029/379A1).

OBJECTIVES

Various tumour diseases are among the leading causes of death worldwide and pose a significant therapeutic challenge. As sensory-vascular-immune interactions mediated by TRPA1 and V1 ion channels and capsaicin-sensitive peptidergic sensory nerve endings may represent potential novel antitumor targets, this work investigated their role in tumour models with varying immunogenicity and vascularisation.

The specific objectives of our work were:

Complex investigation of the role of the capsaicin-sensitive peptidergic sensory nerve system in tumour models with different vascularisation and immunogenicity

1) osteosarcoma

2) breast adenocarcinoma

3) melanoma

mouse models of melanoma by RTX desensitization.

To investigate the complex mechanism of action of the SSAO inhibitor, TRPA1 and V1 antagonist SzV-1287, involved in sensory-vascular-immune interactions in these in vitro and in vivo tumour models.

EXPERIMENTAL MODELS AND TEST METHODS

1. Experimental animals

We used male and female Balb/c and C57Bl/6J mice weighing 20-30 g, 8-10 weeks old. The animals were housed in standard-sized polycarbonate cages (330 × 160 × 130 mm, 5-7 mice/cage or 330 × 100 × 130 mm, 2-5 mice/cage) and fed with standard rodent food (LT/n, Szinbád Kft., Gödöllő) and tap water ad libitum. Housing conditions were maintained at 24-25 °C, 50-60% relative humidity, and a 12:12 h dark-light cycle. All experimental protocols were approved by the PTE MÁB and the ÁTET (permit numbers BA/73/00656-8/2024 and BA02/2000-23/2016), and the experiments were performed by European legislation (Directive 2010/63/EU) and the Hungarian government decree on the protection of animals used for scientific purposes (40/2013, II. 14.).

2. Experimental models

2.1. OS model

K7M2 cells were cultured in DMEM medium at 37 °C in a humidified atmosphere containing 5% CO2. Cells were passaged at 80-90% confluency. Mice (Balb/c) were anaesthetised by intraperitoneal injection of sodium pentobarbital (Euthanimal, Alfasan Netherland BV). After shaving the right hind limb, a small incision was made in the tibia, and a small opening was made using a 27-gauge needle. A suspension of 5×10^5 K7M2 cells (in 10 µL PBS) was then injected into the bone defect using a Hamilton pipette. The sham-operated group received only PBS instead of tumour cells. Following intratibial injection of K7M2 cells, mechanonociceptive threshold, tibial diameter, spontaneous pain behaviour, dynamic weight bearing, and bone structural changes (using micro-CT) were assessed at the respective time points.

2.2 Breast adenocarcinoma

4T1 tumour cells transfected with luciferase (firefly luciferase) were cultured in vitro and prepared for in vivo transplantation. The animals were anaesthetised by intraperitoneal injection, and then 50 µl of 1:1 Matrigel®-PBS solution containing 1×10^6 4T1 cells was injected (orthotopically) into the adipose tissue of the fourth mammary gland of BALB/C mice subcutaneously using a Hamilton syringe (55). During the 9-day experiment, the general condition and weight of the animals were monitored daily. Our experimental setup consisted of RTX pretreatment, injection of 4T1 cells, and monitoring of tumour growth and activity.

2.3 Melanoma model

After anaesthetisation of C57Bl/6 mice, 1×10^5 B16F10 melanoma cells dissolved in sterile HBSS (Hanks balanced salt solution) are injected subcutaneously. After injection of tumour cells, the general condition and weight of the animals were measured every two days. Tumour growth was monitored using an electronic calliper (Mitutoyo, Kawasaki, Japan) and magnetic resonance imaging (MRI).

3. Pharmacological methods

3.1 RTX pretreatment

Long-term desensitisation of capsaicin-sensitive sensory neurons was performed by systemic RTX (Sigma Aldrich) treatment (subcutaneous injections of 10, 20, 70 and 100 mg/kg RTX on four consecutive days). Loss of nerve terminal function was demonstrated by the absence of eye erythema after 10 mL l 0.1% capsaicin was instilled into the eye (41).

3.2 Treatment with SV-1287 and LJP-1207

The effects of SV-127 and the reference SSAO inhibitor LJP-1207 were investigated in 3 tumour models (osteosarcoma, breast tumour and melanoma). The compounds were synthesised at the SE Institute of Organic Chemistry as described previously (48). LJP-1207 was applied in distilled water, and SV-1287 in a vehicle containing 20% Kolliphor HS 15 (polyethene glycol 15-hydroxystearate) and 80% distilled water. Solutions at concentrations of 2 mg/ml and 5 mg/ml were always prepared fresh immediately before administration. The compounds were administered intraperitoneally (i.p.) daily at doses of 20 mg/kg and 50 mg/kg, respectively, based on previously published studies from our institute (54, 56-58). The treatments were administered intraperitoneally, once daily, at least 30 minutes before the respective measurement procedures.

4. Methods

4.1 ATP (adenosine triphosphate)-based cell viability assay

The ATP-based cell viability assay measures the ATP content of metabolically active cells and is therefore suitable for assessing cell viability and cytotoxicity. The CellTiter-Glo® luminescence assay was used to examine cells cultured in 96-well plates after treatment with different concentrations of SSAO inhibitor (SzV-1287), calculating cell viability based on the

luminescence rate. The DMSO concentration used as a control was the same as that of the solvent in the treated samples to ensure a fair comparison.

4.2 Dynamic Plantar Eszthesiometer (DPA)

To measure the mechanonociceptive threshold, a dynamic plantar Eszthesiometer was used, which applied gradually increasing pressure to the hindfoot of the mice. Pain threshold was determined by the aversive reflex, with the force value recorded in grams. Three measurements were taken on each hind limb, and the results were averaged. The change in pain threshold was then calculated as the percentage difference from baseline.

4.3 Dynamic limb load measurement

To assess articular nociception, we used a dynamic weight distribution balance (DWB) meter, which records the weight and surface area loaded by the hind limbs of mice on the floor. The animals were allowed to move freely in the sensory chamber for 5 minutes while the system analysed their movements in real time. The data were manually checked, and the relative loads on the right and left hind limbs were compared as percentages.

4.4 Measurement of spontaneous pain parameters

To assess the behavioural signs of spontaneous pain, mice were placed in Plexiglas cages and observed for 2 minutes after 30 minutes of acclimation. Two types of pain-related behaviours were recorded: the number of flinching reactions (sudden end twitching) and the duration of guarding behaviour (passive retraction of the limb under the body). These voluntary pain responses provide objective information about the level of pain.

4.5 Measurement of tumour size

The anteroposterior and mediolateral diameters of the knees were measured using a digital micrometre, and the change from the preoperative values was calculated as a percentage. Changes in the size of breast tumours and melanomas were monitored daily using a digital measuring device, which recorded the length, width, and height of the tumours. Tumour volumes were calculated using a standard formula, and growth was assessed by the change in size over time.

4.6 Bone structure measurements

In our osteosarcoma experiment, structural changes in the bones of the right proximal tibia were monitored in vivo by micro-CT on days 0, 17 and 28. The study was performed with a voxel

size of 17.5 µm under ketamine-xylazine anaesthesia, and the resulting images were analysed using CT Analyser® software with assignment of identical ROIs. Parameters measured included changes in bone volume and structural characteristics.

4.7 Bioluminescence imaging

After inoculation of transfected 4T1 cells, animals were subjected to bioluminescence imaging on days 1, 3 and 8 using the IVIS Lumina III system. Cells expressing the enzyme "firefly luciferase" emit light after administration of D-luciferin, which is recorded by the imaging system. Images were acquired with uniform settings, and ROIs containing the luminescent signal were automatically selected. Full radiances were used for quantitative analysis.

4.8 Magnetic resonance imaging (MRI)

The growth of breast tumour and melanoma was monitored by MRI imaging on days 2 and 6 for the former and days 12 and 19 for the latter. The study was performed using a Bruker PharmaScan 4.7 T PET MRI machine with a T2-RARE sequence under isoflurane anaesthesia. The 3D Slicer software was used for volume analysis of the MRI images.

4.9 Protease activity and fluorescence analysis of the tumour vascular network

ProSense 680 and AngioSense fluorescence assays were used to investigate the increased protease activity and tumour vascularisation in breast tumours, using the FMT 2000 system. The assays were administered intravenously under general anaesthesia, followed by three-dimensional fluorescence tomography of the debrided area 24 hours later. The signal intensity in the tumour area was automatically analysed by TrueQuant software, and the values were recorded pixel by pixel.

4.10. Radioimmunoassay (RIA) determination of neuropeptide concentrations

The concentrations of CGRP, somatostatin and Substance P were determined from homogenates of breast tumour tissues by radioimmunoassay (RIA). Radioactively labelled peptides, specific antibodies and standards were used for the measurements and samples were incubated at 4 °C for 48-72 hours. The free and bound fractions were separated by centrifugation, and radioactivity was measured by gamma counter; the results are expressed as fmol/mg tissue (SOM-LI, CGRP-LI, SP-LI).

4.11. Histological studies

Glial cell immunohistochemistry

In the osteosarcoma model, activation of central nervous system glial cells was assessed by immunohistochemistry to evaluate pain-related neuroinflammation. We prepared 30-µm sections of perfusion-fixed brain and spinal cord tissue and labelled cells with antibodies against GFAP (astrocytes) and Iba1 (microglia). Quantitative analysis of the stained samples was performed using a Nikon microscope and Neurolucida software according to a modified protocol optimised for nociceptive pathways.

CD3, CD31 and CD45 immunostaining

For the immunohistochemical analysis of tumour tissue in the breast tumour model, we used CD3, CD31 and CD45 antibodies to examine T cells, endothelial cells and immune cell infiltration. Specific primary and peroxidase-conjugated secondary antibodies were used on formalin-fixed paraffin-embedded samples after H&E staining and antigen digestion, and labels were visualised with DAB chromogen. Digitised sections were evaluated using CaseViewer and HistoQuant software, and the expression of each marker was determined as the ratio of masked to annotated areas (rMA, %).

4.12. Statistical analysis

For statistical analyses, an independent samples t-test was used to compare two groups, and a two-way ANOVA with a Bonferroni post hoc test for four groups. For nociceptive tests, a repeated-measures two-way ANOVA was used, along with the Bonferroni correction. Data were tested for normality using the Shapiro-Wilk test. Results are presented as mean ± SEM, and $p < 0.05$ was considered statistically significant.

RESULTS

1. Characterisation of the mouse OS model accounting for gender differences

In the mouse osteosarcoma model, both sexes showed significant mechanical hyperalgesia, reduced limb load and spontaneous pain-related behaviours (flinching, guarding). In contrast, no such changes were observed in the control group. In the tumour limb, pain sensitivity was detectable from day 10 and persisted until the end of the observation period, with a concomitant significant increase in tibial diameter of up to 70-90%. No gender differences were observed in baseline values, the degree of pain, or morphological responses. In osteosarcoma-induced

animals, significant microglial activation (Iba1-positive cell density increase) was observed in the ipsilateral posterior horn of the L4-L6 spinal segment on day 14 in both sexes. This activation was normalised by day 28, and no changes were observed in brain areas (PAG, SSC) at either time point. However, astrocyte activation (as indicated by GFAP positivity) only increased significantly on day 28, suggesting late neuroinflammation induced by osteosarcoma.

2. A complex analysis of the role of capsaicin-sensitive neurons

2. 1 Role of capsaicin-sensitive afferents in OS-induced pain

In an osteosarcoma model, defunctionalisation of capsaicin-sensitive sensory fibres (RTX treatment) did not affect either pain parameters (mechanical hyperalgesia, limb load, spontaneous pain behaviour) or tumour growth. Pain sensitivity and tumour-induced behavioural changes were similar in both RTX-treated and control groups. The morphological features of the tumour and the bone structure also remained unchanged, so no further histological examination was performed.

2.2 Role of capsaicin-sensitive afferents desensitisation in a 4T1 cell-induced breast cancer model

The early phase was associated with a faster increase in tumour volume, although this difference disappeared by later time points and did not become statistically significant by MR-based volumetry. Bioluminescence imaging showed no difference in tumour cell viability between treated and untreated groups. However, vascular permeability was significantly higher in RTX-treated animals, as indicated by the accumulation of AngioSense. Nevertheless, neither intratumour protease activity, nor neuropeptide concentrations (CGRP, SP, somatostatin), nor expression of immunohistochemical markers (CD3, CD31, CD45) showed significant differences between groups. Overall, the knockdown of capsaicin-sensitive nerve endings had a mild effect in accelerating early tumour growth and enhancing vascular leakage.

2.3 Role of capsaicin-sensitive afferents in a mouse model of melanoma

In the B16 melanoma mouse model, damage to capsaicin-sensitive afferent nerve endings (RTX treatment) did not significantly affect tumour growth or vascular network development. Tumour volume and weight evolution in the treatment and control groups showed similar rates during the entire observation period, as determined by both manual and MRI-based measurements. The proportion of CD31-positive areas decreased slightly in the RTX group, but this difference

was not statistically significant. Our results suggest that loss of function of capsaicin-sensitive fibres does not significantly affect melanoma growth or vascularisation.

3. The in vitro and in vivo potential antitumor effects of the SSAO inhibitor compound SzV-1287

3.1 In vitro ATP-based viability assay in K7M2, 4T1 and B16 cell cultures

SV-1287 treatment caused a concentration-dependent decrease in cell viability in all three tumour cell lines tested, as measured by a reduction in luminescent ATP synthesis. The sensitivity of the effect varied by cell type: B16 melanoma cells were more sensitive to lower concentrations, whereas 4T1 and K7M2 cells required higher doses to achieve a significant reduction in viability.

3.2 Investigation of the effects of SzV-1287 treatment in an osteosarcoma model

After the injection of osteosarcoma cells, persistent mechanical hyperalgesia and pain avoidance behaviour were already established from day 8. The treatment with SV-1287 did not result in significant improvements in pain thresholds, limb load or spontaneous pain behaviour compared to the control group, and tumour size did not differ between treatment groups.

3.3. Analysis of SzV-1287 in a mouse 4T1 triple negative breast cancer model

Treatment with SzV-1287 did not significantly affect tumour growth, tumour cell viability or tumour volume, which were similar in both groups. Signal intensities measured by bioluminescence imaging and FMT-based ProSense680 and AngioSense assays showed no difference between the SzV-1287 and solvent-treated groups.

3.4 Testing of SzV-1287 in a B16 melanoma model

During the 21-day trial, the B16 melanoma tumour showed gradual growth; however, treatment with the SSAO inhibitor SzV-1287 resulted in a significant reduction in tumour volume, especially at the 50 mg/kg dose. Tumour volume and mass were significantly lower after treatment with SzV-1287 and LJP-1207, as determined by both digital measurements and MRI-based volumetry. The general condition of the animals was not affected by the treatments, as confirmed by body weight changes.

DISCUSSION, CONCLUSIONS

Our experiments were the first to investigate the complex effects of capsaicin-sensitive sensory nerve endings and our proprietary SSAO inhibitor SV-1287, which plays a role in sensory-vascular-immune interactions, in mouse models of tumours with different vascularisation and immunogenicity - osteosarcoma, mammary adenocarcinoma, and melanoma. In the osteosarcoma (K7M2) model, we observed glial activation indicative of central sensitisation and persistent pain behaviour. Still, desensitisation of capsaicin-sensitive nerve endings did not affect either pain or tumour growth, suggesting that these fibres do not dominate tumour-induced pain phenomena in this model. The development of bone tumour-induced pain, particularly osteosarcoma, is a complex process involving both peripheral and central sensitisation mechanisms (59). Tumour cells release inflammatory mediators, creating an acidic microenvironment that triggers sensitisation of sensory nerve endings and DRG neurons. In addition, activation is observed in the dorsal horn of the spinal cord and brain pain processing regions, which explains why increased pain sensitivity may occur before the tumour is visibly growing (60). Our studies have confirmed that central nervous system glial activation, including early microglial and late astroglial responses, plays a key role in the development and persistence of osteosarcoma pain (61). In contrast, desensitisation of capsaicin-sensitive sensory nerve endings did not affect tumour growth or pain parameters, suggesting a dominance of central sensitisation (62). In contrast, in the 4T1 orthotopic breast tumour model, denervation with RTX resulted in faster tumour growth and increased vascular permeability in the early phase. However, it did not significantly alter tumour size or immunohistochemistry in the long term. There were no significant differences in tumour tissue structure, neuropeptide levels and inflammatory markers, with the initial size difference probably due to peritumoral oedema (63). The difference in tumour size measured by mechanical pusher and MRI is presumably because MRI only considers tumour volume. In contrast, calliper measurement also considers peritumoral oedema, the latter often being pronounced in aggressive breast tumours (64). The early tumour growth and increased vascular permeability observed in this experiment may also suggest a protective, regulatory role of capsaicin-sensitive vascular nerve endings (65). Our results are consistent with previous studies where capsaicin increased the development of lung and cardiac metastases in 4T1 tumours at desensitising doses. In contrast, lower, non-desensitising doses reduced the incidence of lung metastases (66). In conclusion, capsaicin-sensitive sensory nerves influence tumour growth and vascular permeability in the 4T1 orthotopic breast tumour model. The increased vascular permeability observed in tumours from

desensitised mice is likely to result from a disruption of local neurogenic vascular regulation, which may contribute to the disruption of tumour vasculature. However, no significant changes in intratumoral protease expression were observed, nor were tumour tissue neuropeptide levels altered. Histological analysis showed that long-term tumour growth, cellular composition and viability were not significantly affected by desensitisation of sensory nerve endings. Our results, in conjunction with previous data, suggest that capsaicin-sensitive afferents and sensory innervation within the tumour microenvironment exhibit homeostatic and antitumor properties. However, in the B16 melanoma model, neither desensitisation of capsaicin-sensitive afferents nor treatment with SzV-1287 significantly affected tumour growth or vessel formation, which may be attributed to low tumour immunogenicity and rapid proliferation. Since B16 melanoma is a less immunogenic, fast-growing tumour, it is conceivably less sensitive to alterations in neural regulation than, for example, the highly immunomodulated 4T1 breast tumour model. Further research is needed to explore the extent to which these neural mechanisms play a role in the pathophysiology of different tumour types and how they affect the tumour-immune-vascular axis (67). We investigated the effects of our proprietary SSAO inhibitor, SzV-1287, a compound with a complex mechanism of action that involves sensory-vascular-immune interactions, first in vitro in cell culture and then in in vivo tumour models. In in vitro cell viability assays, B16 melanoma cells showed high sensitivity, while 4T1 and K7M2 cells responded only to higher concentrations of the treatment. The results obtained in the osteosarcoma-induced pain model suggest that treatment with SzV-1287 had no measurable effect on either tumour-induced pain behaviour parameters or tumour growth dynamics. The aggressive bone-forming nature of the K7M2 model and the complex tumour-induced nociceptive response may differ from that of soft tissue tumours, and it is also hypothesised that the target molecules of SzV-1287 are expressed or activated differently in this tissue environment. A further possible explanation is that the pain caused by bone tumours is not primarily mediated through capsaicin-sensitive afferent fibres, so that TRPA1/TRPV1 modulation alone is not sufficient to alleviate symptoms. Several studies have shown that VAP-1 expression is associated with various tumours, including metastatic lesions of the colon, stomach, and lung (68,69). In our experiments, we also investigated the effect of SzV-1287 on tumour proliferation and viability in an orthotopic 4T1 breast adenocarcinoma mouse model. Due to the rapid division of injected 4T1 cells, the tumour showed a significant increase in volume by day 9 in both the solvent- and SzV-1287-treated groups. Although a smaller mean tumour volume was observed with the SzV-1287 treatment, the difference did not reach statistical significance. These results are in line with previous literature suggesting that due to

the complexity of the tumour microenvironment and the different sensitivity of different tumour models, drug effects may vary significantly between models. In our B16 mouse melanoma model, we were the first to demonstrate the tumour-reducing effect of SzV-1287. The results showed that a higher dose of SzV-1287 (50 mg/kg) resulted in a significant reduction in tumour volume compared to the control group, which was confirmed by both digital volumetry and MRI scans. The mechanism of the antitumour effect is partly based on the inhibition of the SSAO enzyme. Still, other effects, such as TRPV1/TRPA1-mediated or anti-inflammatory effects, may also be involved in the case of SzV-1287. Several studies have demonstrated its role in a B16 melanoma mouse model using anti-VAP-1 monoclonal antibodies and small-molecule VAP-1 inhibitors. VAP-1 inhibitors were able to slow down the growth of melanomas and lymphomas and reduce tumour neoangiogenesis (70). The identification of the VAP-1/SSAO system as a tumour-specific target and the selective modulation of capsaicin-sensitive afferents offer the possibility of developing new therapeutic strategies. Our results suggest that the role of sensory innervation and SSAO inhibition is strongly dependent on tumour type, immunogenicity and microenvironment. Thus, their targeted modulation may form the basis of personalised antitumor approaches.

SUMMARY OF NEW RESULTS

I. Using a K7M2 osteosarcoma mouse model, we analysed in detail the development of bone tumour-induced pain, showing central sensitisation (spinal microglia and astroglia activation). At the same time, desensitisation of capsaicin-sensitive nerve endings did not affect either tumour growth or pain parameters. In a 4T1 breast carcinoma model, desensitisation initially resulted in faster tumour progression and increased vascularisation, suggesting that capsaicin-sensitive afferents may play a protective role in regulating the tumour microenvironment. Eradication of capsaicin-sensitive sensory nerve endings did not affect melanoma growth or vascularisation, which may be related to lower tumour immunogenicity and sensory nerve insensitivity.

II. We investigated the effects of our proprietary SSAO inhibitor SV-1287, which has a complex mechanism of action involved in sensory-vascular-immune interactions, in in vitro and in vivo tumour model systems. In vitro, the decrease in ATP synthesis reflected a reduction in cell viability in all tumour types, particularly in the B16 melanoma cell line, showing significant sensitivity even at relatively low concentrations of 50 μM. This confirms previous in vivo results where SV-1287 resulted in tumour volume reduction in this model. In 4T1 breast carcinoma cells, a significant decrease in viability was only observed above 250 μM. This result

may be of particular relevance for models investigating sensory nerve-tumour interactions, where the effects of SV-1287 are hypothesised to be partly mediated through non-tumour cell-specific mechanisms. Here, in vivo treatment did not lead to a significant reduction in tumour volume, but the different immunogenicity and microenvironmental sensitivity may explain the varying response. The osteosarcoma cell line K7M2 exhibited intermediate sensitivity to SV-1287, as evidenced by a significant reduction in cell viability at concentrations above 100 μM. In our in vivo studies, SV-1287 showed neither analgesic nor antitumour effects in this model. Our studies offer a new perspective on defining the tumour microenvironment and its neurovascular-immune components, to identify promising new therapeutic targets.

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CONGRESS POSTER PRESENTATIONS

Noémi Bencze, Bálint Scheich, András Adámy, Nikolett Szentes, Éva Borbély, Éva Szőke, Imola Wilhelm, Zsuzsanna Helyes: Complex examination of cancer pain and glial cells in the brain in osteosarcoma murine model. V Interdisciplinary PhD Conference, Pécs, 27-29 May 2016.

Noémi Bencze, Bálint Scheich, András Adámy, Nikolett Szentes, Éva Borbély, Éva Szőke, Imola Wilhelm, Zsuzsanna Helyes: Complex examination of tumour pain and brain microglial activation in a mouse osteosarcoma model. Joint Scientific Conference of Hungarian Pharmacological, Anatomical, Microcirculatory and Physiological Societies (FAMÉ 2016 June 1-4).

Bencze N., Horváth Á., Tékus V., Szentes N., Scheich B., Bölcskei K., Szőke É., Mócsai A.,Tóth SarudyÉ., Mátyus P., Pintér E., Helyes Zs.: The role of SEMICARBASIDE-SCENCYTHYL AMINOXIDE GATE, SZV -1287 in microglial activation in a mouse model of chronic arthritis. Hungarian Society of Experimental and Clinical Pharmacology, Drug Innovation Congress, 7-9 May 2017.

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