In vitro investigations of the role, signaling and effects of novel anti-inflammatory, analgesic, and anti- cancer drug targets

Doctoral (PhD) Thesis

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Abbreviations	Meaning
AITC	Allyl isothiocyanate
ATRA	All-trans retinoic acid
CAPS	Capsaicin
CGRP	Calcitonin gene-related peptide
CHO	Chinese hamster ovary
CIBP	Cancer-induced bone pain
CLs	Intrastrand crosslinks
CPM	Count per minute
CRABPII	Cellular retinoic acid binding protein 2
Cy3	Cyanine 3
Cy5	Cyanine 5
CZP	Capsazepine
DAPI	4′,6-diamidino-2-phenylindole
DDR	DNA damage response
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DRG	Dorsal root ganglion
DSB	Double-strand break
FBS	Fetal bovine serum
Gapdh	Mouse glyceraldehyde 3-phosphate dehydrogenase gene
GDP	Guanosine diphosphate
GIRK	G-protein inward rectifying potassium channels
GPCR	G-protein coupled receptor
GTP	Guanosine triphosphate
H&E	Hematoxylin-eosin
HC	HC-030031
HR	Homologous recombination
H_2O_2	Hydrogen peroxide
ICLs	Interstrand crosslinks
MAPK/ERK	Mitogen-activated protein kinase/ extracellular signal regulated kinase
MSCS	Mesenchymal stem cells
NHEJ	Non-homologous end joining
OS	Osteosarcoma
RAR	Retinoic acid receptor
RAREs	Retinoic acid response elements
RLU	Relative light units

ROS	Reactive oxygen species
RT	Room temperature
RXR	Retinoid x receptor
SCLC	Small cell lung cancer
SSB	Single-strand break
SST	Somatostatin
SSTs	Somatostatins
SST_2	Somatostatin receptor 2
SST_4	Somatostatin receptor 4
TRP	Transient receptor potential
TRPV1	Transient receptor potential vanilloid 1 protein
TRPV1	Human transient receptor potential vanilloid 1 gene
Trpv1	Mouse transient receptor potential vanilloid 1 gene
TRPA1	Transient receptor potential ankyrin 1 protein
TRPA1	Human transient receptor potential ankyrin 1 gene
Trpa1	Mouse transient receptor potential ankyrin 1 gene
VGCC	Voltage gated calcium channels

Table of Contents

Background of the present work, general introduction

1. Inflammation and pain as unmet medical need conditions

Inflammation is the response of the body to harmful stimuli, such as infection and tissue damage, to remove these stimuli and heal the injury by different vascular, immune and neural cellular mechanisms [1]. This process is important to restore the homeostasis, but persistent inflmmation beyond the need lead to chronic painful conditions and variety of chronic inflammatory diseases [2]. The complexity of the inflammatory response is mediated by a broad range of cell types including immune cells, neurones (primary afferent neurons in sensory ganglion, and secondary sensory neurons in the spinal cord), and other non-neuronal cells (epithelial, endothelial, fibroblasts and keratinocytes) [3].

Pain as defined by the International Association for the Study of Pain is ‘‘an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage’’ [4]. Therefore, it provides an important alert and defense mechanism, but pain can last longer and become chronic substantially decreasing patients’ quality of life [5]. Pain can be classified into nociceptive pain, induced by activation of nociceptors upon specific stimuli that arises from tissues damage and inflammation, and neuropathic pain, caused by damage or injury of somatosensory nerves [6]. Nociceptive pain is usually associated with inflammatory diseases such as in arthritis [7], whereas neuropathic pain is associated with nerve damage such as in diabetes [8]. Current pain medications including nonsteroidal antiinflammatory drugs, opioids, and adjuvant analgesics like certain anticonvulsants and antidepressants have limited efficacy and cause a variety of side effects such as gastrointestinal, kidney and bone marrow damage, sedation, dizziness [9]. Furthermore, these analgesics are only effective in treating chronic pain with neuropathic mechanisms. Unfortunately, there has been no bearkthrough in the field of analgesics in the last centuries, therefore, there is a huge need to develop novel drugs with substantially different molecular mechanisms.

2. Neurogenic inflammation and the capsaicin-sensitive peptidergic nerves

Capsaicin-sensitive neurons are a subdivision of nociceptive sensory neurons that consist of small, myelinated Aδ- and unmyelinated C-fibers. These neurons were characterized as capsaicin-sensitive afferents” as they respond to capsaicin (8-methyl-Nvanillyl-6-nonenamide) the pungent

ingredient in chili peppers (Capsicum anuum) [10]. This response occurs through the transient receptor potential vanilloid 1 (TRPV1) [11]. Capsaicin-sensitive nerve terminals are special because they have three different functions. First, as afferent neurons, they play a role in nociception (afferent function). Second, these nerve endings induce the so called ‘’neurogenic inflammation’’ mediated by pro-inflammatory neuropeptides, for example tachykinins (substance P, neurokinin A) and calcitonin gene-related peptide (CGRP) causing arteriolar vasodilatation and venular plasma protein extravasation in the innervated areas [12], [13]. On the other hand, it has been recognized that during the local neurogenic inflammation, an anti-inflammatory and analgesic neuropeptide such as somatostatin (SST) are also released from capsaicin-sensitive afferent neurons [14], [15]. This SST was found to exert local interaction with the inflammatory mediators, and moreover, reach distant parts of the body where it acts as systemic anti-inflammatory agent, [16], [17] a phenomenon that was described as the “sensocrine” function of these afferent neurons [10], [18]. It was later shown that low frequency electrical stimulation of nociceptors caused elevation of SST level in the plasma leading to maximal anti-inflammatory effect without any pain sensation [19]. In addition, it was found that these capsaicin-sensitive afferents can be activated by the transient receptor potential ankyrin 1 (TRPA1) ion channels which found to be co-expressed with TRPV1 [20]. These findings suggested a possible role for these nerve endings in diseases (inflammatory diseases, pain conditions and cancer pain, neurodegenerative diseases) and contributed to the pharmacological development of novel analgesics and anti-inflammatory drugs.

3. Transient receptor potential (TRP) channels

TRP channels are a superfamily of 28 cation channels. The mammalian TRP family is divided into six subfamilies based on sequence homology (Figure 1). These subfamilies are TRPA (ankyrin) TRPC (canonical), TRPM (melastatin), TRPML (mucolipin), TRPP (polycystin) and TRPV (vanilloid) [21]. Based on their sequence and topological variations, these families can be classified in two groups: group 1 channels, which includes TRPV, TRPM, TRPC and TRPA, and group 2, includes TRPML and TRPP [22]. All members of the TRP channels superfamily consist of six transmembrane helices, permeable to cations, and have different levels of sequence homology. However, unlike other families of ion channels, TRP channels express an interesting diversity of cations selectivity and specific activation mechanisms [23].

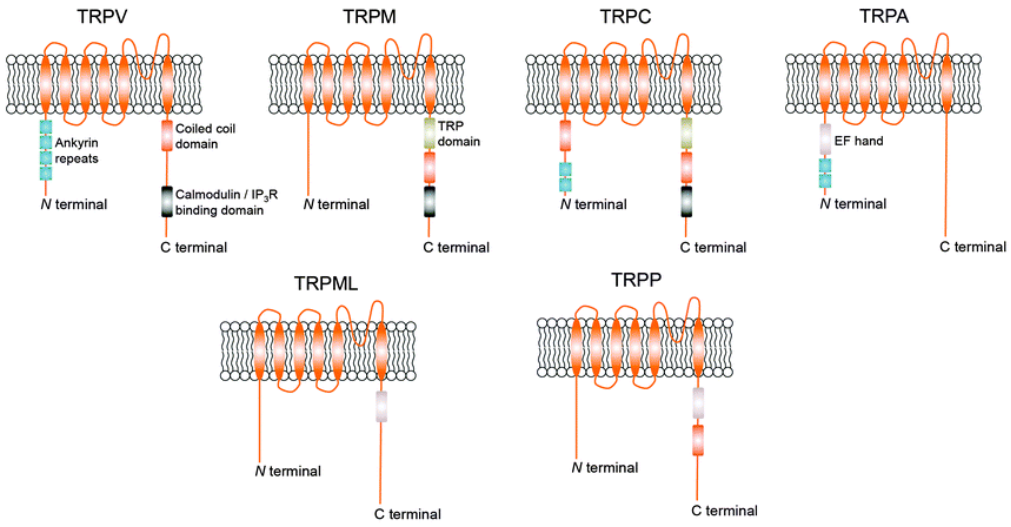

Figure 1. TRP channel subfamilies and structure organization. Adapted from (Rodrigues et al., 2016) [24]

TRP channels are nonselective cation channels that, once active, can increase intracellular concentrations of cations such as Ca^{2+}, Na^{+}, and Mg^{2+}, but they are popular for Ca^{2+} homeostasis [25]. Since expression of TRP channels mediates ion homeostasis, they play vital role in cell biology such as apoptosis, cell proliferation, and migration [26]. They are also important in other physiological processes such as insulin secretion and vascular tone regulation [27]. Therefore, changes in TRP channel expression could be involved in different disorders. So far, most of the alterations in TRP proteins are not caused by genetic mutations, but epigenetic alterations leading to decreased or increased levels of expression that are believed to be causative factors of various pathophysiologies [28]. Different TRP proteins were shown to function as useful markers in predicting cancer stage hence may be promising targets for pharmaceutical treatment. TRP channels are expressed in sensory neurons and shown to play an important role in cancer-related pain [29]. The physiological functions of TRP proteins in cancer are not fully understood and may differ depending on cell type and channel subtypes. Hence, these channels require intensive efforts for drug discovery and development.

Several exogenous irritants, painful stimuli, endogenous activators, temperature, and pressure induce TRP channels activation. Some of these channels can sense hot or cold and are proposed to function as the body's thermometers. Other TRP channels act as sensors that are activated mechanically or osmotically [21]. Examples of endogenous modulators are the inorganic ions (Ca^{2+} and Mg^{2+}) and bioactive lipids (eicosanoids, anandamide). Different exogenous natural

products such as capsaicin, menthol and synthesized small molecules have been shown to affect TRP channel activation [30].

4. TRP channels in pain, inflammation, and cancer

TRP channels have been associated with noxious sensation, including TRPV3, which is sensitive to farnesyl pyrophosphate and is involved in the sensitivity to noxious heat [31], TRPV4, expressed in primary afferent nociceptive nerve fibers and acts as osmo-transducer [32], TRPC1 and TRPC6, which work together with TRPV4 in the mediation of hyperalgesia induced by inflammatory mediators [33], TRPC4 and TRPC5, which together are relevant for pain hypersensitivity and neuropathic pain [34], [35], TRPM3, expressed in a number of small sensory neurons in dorsal root and trigeminal ganglia and involved in the nocifensive response to heat [36], and TRPM8, plays a role in cold hyperalgesia and tactile allodynia [37]. However, the role of these channels in pain transmission and detection is less characterized than that of TRPV1 or TRPA1 [38].

Despite all recent improvements in cancer treatment, the chances of failure, reoccurrence and death are still high. Metastasis is a major hallmark of cancer characterized by the migration and invasion of cancer cells from its primary location to other organs. Metastasis converts cancer from being curable by surgical excision to one that needs chemotherapy and may eventually causes death. Drug discovery studies to target TRP channels have expanded to include new diseases such as asthma, obesity, chronic itch, overactive bladder, anxiety, and cancer. Nowadays, there is a growing body of evidence regarding the role of TRP channels in cellular proliferation, abnormal differentiation, growth, and invasion of cancer, all associated with Ca^{2+} homeostasis [25], [26]. Studies showed that altered expression of TRP channels is associated with tumor progression to late stages in several types of cancers. TRPs have been involved in breast, prostate, bladder, and kidney cancers as well as in melanoma and glioma [39]. The first study investigating TRP channels in sarcomas showed an overexpression of TRPM8 in osteosarcoma (OS) cells and knockdown of these channels decreased the proliferation and malignant progression of OS cells and facilitated epirubicin-induced apoptosis [40]. Another study showed a significant increase of TRPM8 subfamily expression in higher clinical stage OS patients compared to those in a lower stage and absence of metastasis [41]. An aberrant expression of TRPC4/C1 subfamily in human synovial sarcoma cell lines has been reported with a deleterious effect upon activation [42]. It has also been

shown that TRPM, TRPC and TRPV are highly expressed in human and murine OS cell lines [43]. Moreover, TRPA1 and TRPV1 have been proposed to play roles in various types of tumors, such as breast, digestive, gliomas, lung, prostate, human and neck squamous cell cancers including oral squamous cell carcinoma [44].

In this thesis, we only address two members of the TRP family, TRPA1 and TRPV1.

5. Somatostatin

Somatostatin (SST), also known as somatotropin release-inhibiting factor is a neuropeptide that has multiple biological effects in many cells and organs throughout the body. It is produced by specific endocrine, immune, gastrointestinal, neuronal cells, and by certain tumors [45]. SST can act locally in a paracrine or autocrine fashion as well as a neuro modulator, or through the blood stream as an endocrine hormone [46]. SST undergoes tissue-specific enzymatic degradation to yield either SST-14 or SST-28 (Figure 2). This is followed by rapid enzymatic degradation reducing or rendering them completely inactive. Therefore, these natural somatostatins (SSTs) have a short half-life of less than 3 minutes [47].

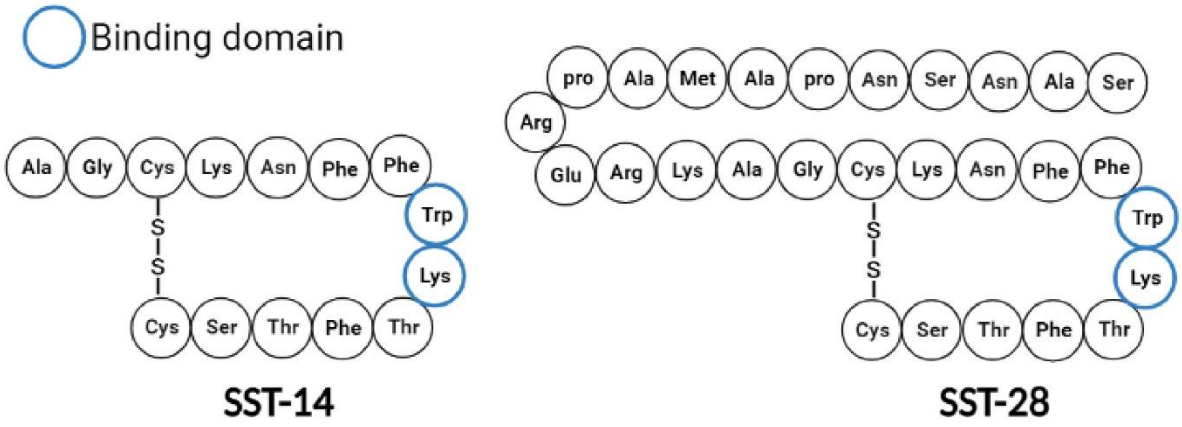

Figure 2. The amino acid sequence of SST. This figure was created with Biorender.com.

SST exerts its biological effect via five specific receptors (SST_1, SST_2, SST_3, SST_4, and SST_5) belonging to the seven transmembrane inhibitory $G\alpha_{i/o}$ protein-coupled membrane receptor (GPCR) family. These receptor subtypes are widely expressed in several tissues including gastrointestinal tract, retina, and brain and may coexist in one cell. They also share structural features and signaling pathways and are encoded by five different genes segregated on chromosomes 4, 17, 22, 20, and 16, respectively. SST2 exist in two isoforms (SST_{2A} and SST_{2B})

that are products of alternative splicing and differ in the length of their carboxyl terminus [48]. Based on structural similarities, these five receptors have been classified into two different groups. The SRIF1 group, including receptor subtypes SST_2, SST_3, and SST_5, and the SRIF2 group, consists of subtypes SST_1 and SST_4 [49], [50]. Many studies demonstrated that receptors in the SRIF1 group exert endocrine and antiproliferative effects, whereas the SRIF2 group, mainly the SST_4 receptor mediate antinociceptive and anti-inflammatory actions without the endocrine effects [51].

The heterotrimeric G-proteins consist of α-, β- and γ-subunits. The α- subunit hydrolyses guanosine triphosphate (GTP) and is structurally and functionally homologous to other GTPases. The β- and γ-subunits form a tightly bound complex and act as one functional unit. There is more than one subtype for each of these subunits. At basal state, the three subunits as well as the α-bound guanosine diphosphate (GDP) - are associated. SST binds and activates SST receptor, which in turn interacts with the G protein leading to the dissociation of GDP from the α-subunit which is then replaced by GTP. Interaction of GTP with α-subunit causes conformational changes that in turn break the α-subunit and the $\beta\gamma$-complex. The separated subunits are then able to interact with further effector proteins modulating several downstream second messenger systems [52]. Activation of SSTs inhibits adenylyl cyclase enzyme, which in turn reduces cAMP and intracellular Ca^{2+} levels, leading to reduction in cell proliferation and in secretion of signaling molecules [48]. The response of G- protein to ligands often diminishes over seconds or minutes. SSTs are phosphorylated and internalized into clathrin-coated vesicles and transported to endosomes before being recycled to the plasma membrane or targeted by the proteasome pathway [53].

6. Somatostatin in pain

Beside the inhibitory effect on cell proliferation and hormone secretion, SST has an important role in pain and inflammation [54]. SST receptors are widely distributed in the central and peripheral nervous systems. Several tissues in the body are innervated by peptidergic sensory neurons, which include myelinated Aβ, Aδ fibers, and unmyelinated C fibers [10]. These fibers transmit pain by signal transmission to the central nervous system. Upon noxious stimuli, a signal from the capsaicin-sensitive sensory neuron to a secondary neuron in the dorsal horn is transmitted by releasing pro-nociceptive and pro-inflammatory neuropeptides such as substance P, and CGRP

triggering pain and neurogenic inflammation [55]. In addition to that, analgesic and anti-inflammatory peptides like SST and opioids are released (Figure 3). These peptides can reach the systemic circulation where they exert their effects in the periphery (sensocrine function). Therefore, the capsaicin-sensitive sensory neurons have a dual function including both the enhancement and attenuation of nociception [10].

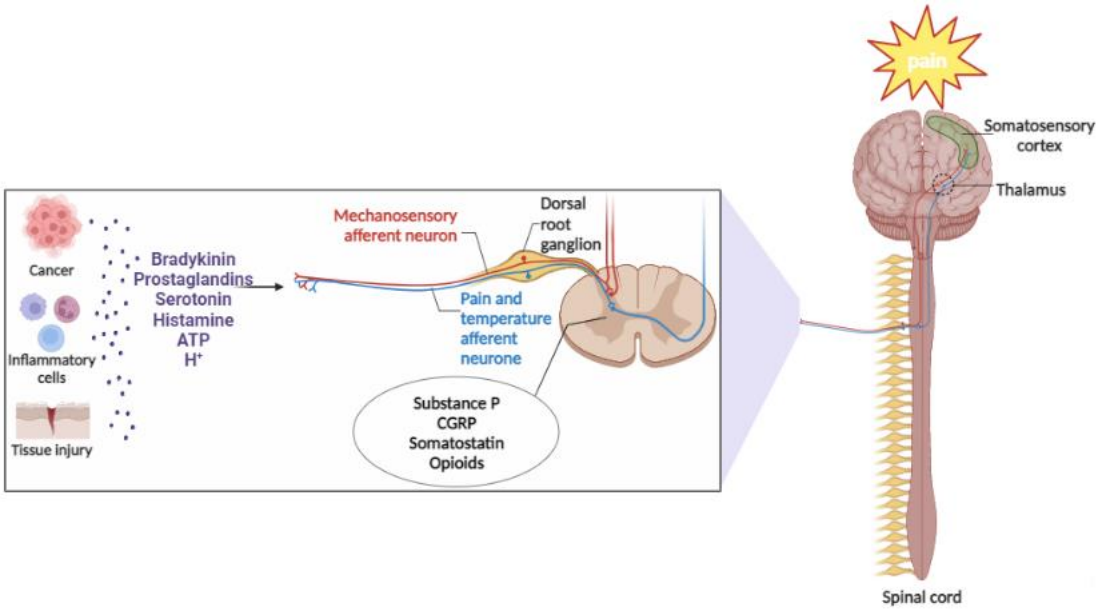

Figure 3. Schematic diagram of pain pathway. This figure was created with Biorender.com.

Previous studies confirmed that endogenous and central release of SST results in antinociception and analgesia by inhibiting spinal neurons processing and transmission of signals. *In vivo* studies revealed inhibition of heat-evoked responses after microinjections of cats with SST into the periaqueductal grey matter and the nucleus raphe magnus [56]. In addition, SST significantly increased pain threshold when microinjected into rat's caudate putamen [57]. Based on in vivo data, SST was proposed to be effective in reducing different pain states, such as cancer pain and inflammatory conditions [58]. For example, intrathecal administration of the SST_2 agonist, octreotide decreased neoplastic pain in cancer patients similar to opioids [59]. Moreover, subcutaneous injection of the same compound provided significant relief of migraine to a level where some patients became pain free [60].

7. Retinoids

Retinoids are a chemical group of compounds, which includes vitamin A [61]. They are important micronutrients which are consumed as dietary intake, either in the form of carotenoids from vegetable sources, or retinol and retinyl esters from animal sources [62]. The liver is a rich

source of retinoids, and the ancient Egyptians used it as a cure for night blindness, which is a symptom of retinoid deficiency [63]. Retinoids play an important role in cell differentiation, proliferation, and apoptosis [64]. Therefore, they have been reported extensively as chemopreventive and chemotherapeutic agents [65]. However, their use has been limited due to a lack of understanding of their complex signaling pathways and their severe adverse effects [66], [67].

8. Osteosarcoma

The bone consists of four main cell types: osteoblasts, osteoclasts, osteocytes, and bone lining cells. Osteoblasts are derived from mesenchymal stem cells, they release new unmineralized bone tissue rich in type I collagen called osteoid that is later mineralized to form mature bone. Osteoclasts originate from hematopoietic progenitors and are responsible for aged bone resorption. Osteoblasts entrapped within the osteoid matrix differentiate into osteocytes that function to balance bone formation and resorption. Some osteoblasts become bone lining cells that line the external surfaces of the bone [68], [69]. Based on shape, the four general categories of bones are flat bones (e.g., skull), long bones (e.g., clavicles, femurs, tibiae), short bones (e.g., carpal bones of hands, tarsal bones of the feet), and irregular bones (e.g., vertebrae) [70].

Throughout life, bones undergo either longitudinal or appositional growth. Longitudinal growth occurs at the growth plate during which the cartilage proliferates in the epiphyseal and metaphyseal areas and then goes through mineralization to form primary new bone. Bones continue to grow in length during childhood and adolescence with the rate of growth influenced by hormones [71]. When the chondrocyte proliferation and cartilage growth cease in the epiphyseal plate, usually at age of 20, bones replace the cartilage and can no longer grow in length [72]. Appositional growth can continue even after longitudinal growth ceases where bones are increasing in thickness and diameter [73].

OS is a primary malignant tumor of the skeleton that mainly occurs near the metaphysis of long bones, particularly the distal femur and proximal tibia. It is derived from the mesenchymal stem cells (MSCs) committed to osteogenic lineage and believed to originate at some stage in the process of differentiation of MSCs to pre-osteoblast or from osteoblast precursors. OS is often fatal in both children and adults and metastatic complications are the major cause of OS-related death [74], [75].

Based on histological characterization, the World Health Organization now reclassifies OS into six subtypes: OS not otherwise specified (conventional OS, telangiectatic OS and small cell OS), low-grade central OS, parosteal OS, periosteal OS, high-grade surface OS, and secondary OS [76]. Tumor grades represent the degree of differentiation of cancerous cells, which reflects the resemblance of these cells to normal cells. Low grade tumors are usually less aggressive, well differentiated and have better prognosis [77], [78]. Therefore, the choice of OS treatment depends on tumor grade. Most of the research is conducted on the conventional subtype, a high-grade tumor that includes chondroblastic, fibroblastic and osteoblastic variants comprise approximately 85% of all OS cases in childhood and adolescence [78].

General research objectives, overall goals

This dissertation includes three different studies where commonly used *in vitro* cell-based assays were applied to examine the toxicity of novel retinoids as preliminary study for future investigations of their ability to activate TRPV1 and/or TRPA1 channels, to test the functional expression of TRPV1 and TRPA1 in OS cell line, and to validate the SST_4 receptor as the target of novel analgesic and anti-inflammatory drug candidates. Thus, the aim of this dissertation is to understand the complex mechanisms behind toxicity and/or efficacy of different synthetic compounds in pain and cancer. The main goals of my PhD work were:

I. Evaluating the cytotoxic and genotoxic potential (safety) of novel retinoic acid derivatives by *in vitro* ATP luminescence-based viability and comet assays in comparison to ATRA on Chinese hamster ovary (CHO) cells.

II. Testing the expression of *TRPA1/Trpa1, TRPV1/Trpv1* mRNA in human and mouse OS tissues by RNAscope as well as testing the functional expression of these channels in OS K7M2 cells using polymerase chain reaction (PCR) gel electrophoresis, RNAscope, $_{45}Ca^{2+}$ uptake, and ATP viability assay in response to their agonists to determine their potential as novel therapeutic targets.

III. Testing the activation and cAMP-related signaling of the SST_4 receptor by potential novel analgesic and anti-inflammatory candidates patented by our group which were suggested to act via the SST_4 receptor compared to two reference SST_4 agonists J-2156 and TT-232.

I. DNA damage induced by novel diphenylacetylene-based retinoids in CHO cells

1. Background

1.1 All-trans retinoic acid (ATRA)

ATRA, the major metabolite of vitamin A isomerases in the body to 9-cis-retinoic acid, and 13-cis-retinoic acid or catabolizes to polar metabolites [79]. ATRA and its isomers have different roles in biological processes during both embryogenesis and adult life [80]. Therefore, it is used either alone or as maintenance therapy in the treatment of various skin diseases and as an anti-cancer drug [81]. Nevertheless, ATRA is unstable and susceptible to isomerization and oxidation, which affects its activity and selectivity [80], [82]. These effects could be of special interest when studying its toxic effect on normal, non-tumor cells. Hence, more stable synthetic derivatives are needed to overcome these problems and offer potential valuable alternatives as pharmaceutical agents with lower toxicity.

The structure of ATRA consists of a bulky lipophilic cyclohexenyl ring, a conjugated side chain, and a hydrophilic carboxylic acid end [61] (Figure 4). These groups play a critical role in retinoid functions. Therefore, several retinoic acid analogs have been previously synthesized such as acitretin, etretinate, and adapalene [83].

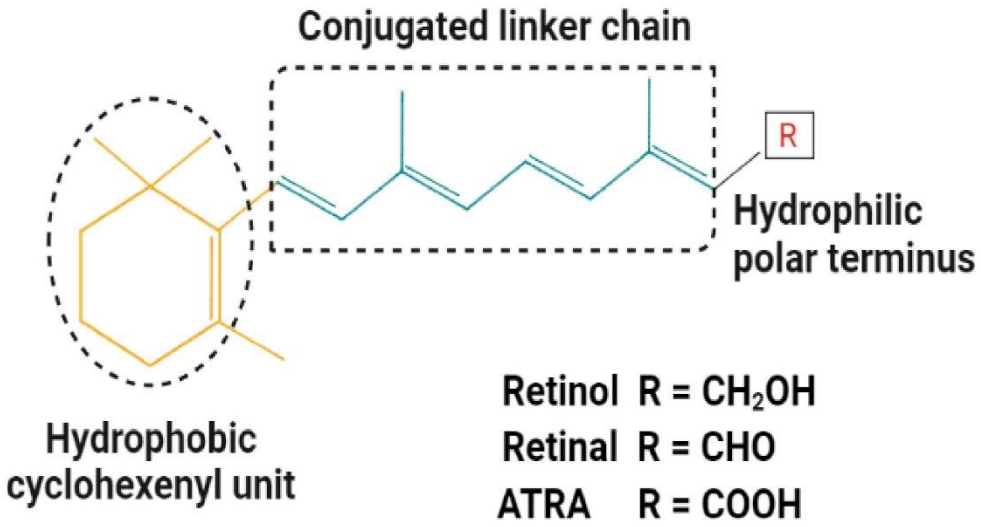

Figure 4. Chemical Structure of retinoids. This figure was created with Biorender.com.

1.2 ATRA mechanism of action

In the cells, ATRA and its isomers bind to cellular retinoic acid binding protein 2 (CRABPII) [84]. CRABP II is a low-molecular weight protein responsible for the translocation of retinoic acids from the cytoplasm to the nucleus [85]. In the nucleus, retinoic acids act by binding to the nuclear retinoic acid receptors (RARs) and retinoid x receptor (RXR) forming RXR-RXR, RAR-RXR homo- or heterodimers [86]. These dimmers bind to retinoic acid response elements (RAREs) in the promoter regions of the target genes leading to conformational changes that release co-repressors and recruit co-activators [87] (Figure 5).

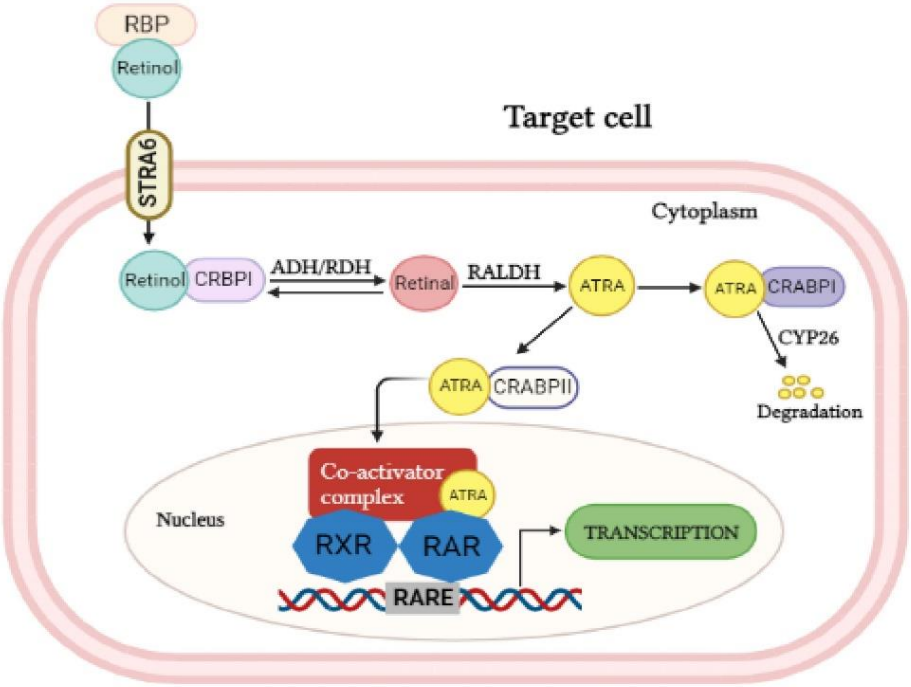

Figure 5. Schematic presentation of the retinoic acid signal transduction pathway. This figure was created with Biorender.com.

There are six different retinoic acid receptors mediate the action of retinoids in the nucleus, the retinoic acid receptors (α, β and γ), and retinoic x receptors (α, β and γ) [88]. The concentrations of retinoids together with the expression of specific receptor iso-type affect the action of retinoids [89].

1.3 DNA damage

It is known that DNA, the genetic material that makes our functional unit of inheritance is very important for cell functions as it produces the necessary cellular proteins [90]. But DNA is

continuously attacked by reactive molecules and thus susceptible to different endogenous and exogenous modifications that sometimes cause mutations and cancers [91]. Also, DNA polymerases that are responsible for DNA replication and repair may encounter errors leading to DNA damage [92]. However, to sustain genome integrity cells have a complex system of repair proteins that engage the appropriate pathway to prevent harmful consequences of DNA damage named as DNA damage response (DDR) which includes cell cycle checkpoints, DNA repair, DNA damage tolerance, and cell death [93] (Figure 6).

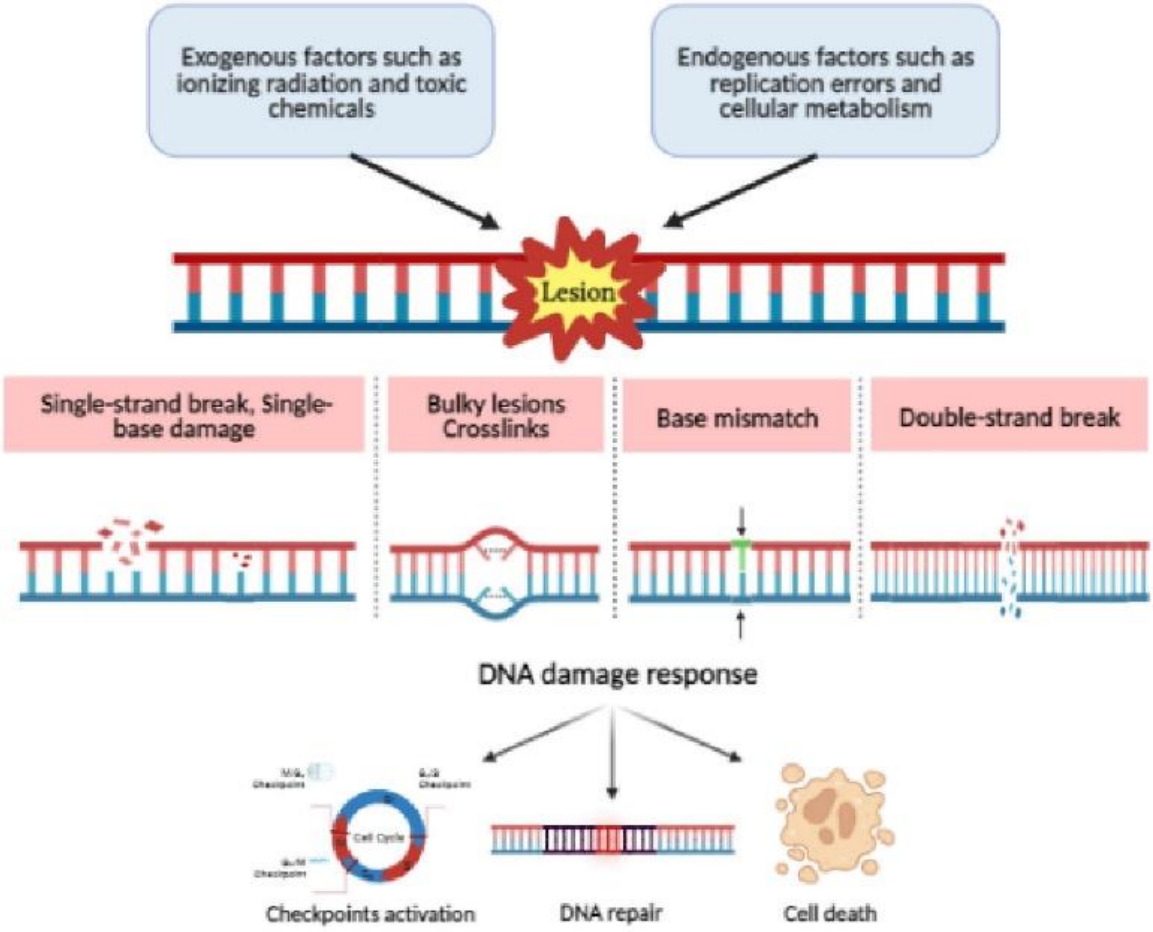

Figure 6. DNA damage response. This figure was created with Biorender.com.

DNA damage can be caused by endogenous or exogenous factors. The endogenous DNA damage occurs upon the hydrolytic reactions of DNA with surrounding water or the oxidative reactions with reactive oxygen species (ROS) [94]. Replication errors, spontaneous base deamination, abasic sites, oxidative DNA damage, and DNA methylation are widely known causes of endogenous DNA damage [91]. This type of damage is the main cause of different hereditary diseases. Exogenous DNA damage is caused by exposure to environmental chemical or physical agents that

have the potential to damage DNA. Examples include alkylating agents, ionizing radiation, UV light, and crosslinking agents [95]. Both endogenous and exogenous DNA damage lead to alteration in DNA structure causing mutations and chromosomal abnormalities [96]. If unresolved, the replication process will be delayed or completely blocked which later causes DNA fragmentation by endonucleases, and cell death [97]. There are two types of DNA fragmentation, single-strand breaks (SSBs), and double-strand breaks (DSBs) [98]. Multiple techniques are developed to determine the presence of DNA fragmentation in cells, such as single cell gel electrophoresis (Comet assay) [99]. SSBs are the most common type of DNA lesions [100]. Endogenously, they are generated in the cells due to oxidative DNA damage, abasic sites, or by action of topoisomerase 1 enzyme that helps to relax the superhelical tension of DNA during replication [101]. SSBs lesion can also form as a result of exogenous toxins such as chemotherapy, and ionizing radiations [102]. The repair mechanism of SSBs differs according to the type of break (long patch or short patch). Briefly, the repair can be done in four steps: DNA damage binding, DNA end processing, gap filling, and ligation to restore the integrity of the phosphodiester backbone [103]. If unresolved, SSBs blocks DNA replication and transcription, and causes the release of apoptosis inducing factor. In addition, they can be converted to double-strand breaks causing genetic instability and high incidence of cancers [101], [104]. These breaks can be detected in the lab by applying one of different methods such as alkaline unwinding [105], alkaline filter elution [105], [106], and comet assay [107]. DSBs arise when the phosphodiester backbone of the two strands of DNA is broken [108]. Therefore, they are more toxic than SSBs. Exogenous exposure to chemical and physical agents as well as endogenous replication, recombination, and repair processes can lead to DSBs [109]. Numerous methods are available to study DNA DSBs. These include neutral filter elution [110], immunofluorescent staining phosphorylated histone H2AX [91], pulsed field gel electrophoresis [110], and comet assay [107]. Another type of DNA damage is the generation of alkali labile sites, in which different cellular processes are involved such as ROS reactions, DNA replication, DNA repair, and base hydrolysis [111]. This type of DNA damage can be evaluated by different methods including the alkaline comet assay [112]. Interstrand crosslinks (ICLs, between bases on the same strand), intrastrand crosslinks (CLs, between bases of the two complementary strands) [113], DNA-protein crosslinks [114], and bases monoadducts [115] are DNA lesions that can also contribute to hereditary disorders and predisposition to cancer [113]. Exposure to crosslinking agents such as cisplatin, psoralen, and

nitrogen mustards leads to the formation of ICLs. Some of these agents are being used either alone or in combination with other agents as effective anticancer therapy [116], [117]. ICLs and CLs can block the replication process imposing a major threat to genome stability. If not repaired, they will cause cell cycle arrest and later cellular apoptosis [118].

1.4 DNA repair

Changes in DNA structure have several effects on replication, transcription, and other processes. When DNA is damaged, a specific chromatin-associated protein network is initiated to sense and repair the damage by DDR [119].

Different DNA repair mechanisms remove DNA lesions, which could severely affect the replication and transcription processes and lead to mutations [120]. The mechanism of repair can be direct or complex. In the first, the lesions are directly removed such as the simple reversal of alkylated bases by the enzyme O^6alkylguanine transferase. In the complex mechanism, specific polymerases and exonucleases are recruited to repair the damage [121]. SSB and DSB repair, base excision repair, nucleotide excision repair, mismatch repair, and ICL repair are different types of complex DNA repair mechanisms [91].

Complex repair mechanism starts with recognition of damaged DNA strand by specific enzymes that recognize the abnormal bases, and then specific exonucleases remove the damaged part of the DNA strand leaving a gap, which will be filled by DNA polymerase that synthesizes a new complementary nucleotide sequence. Finally, DNA ligase seals the strand break to have an intact DNA molecule [122].

If not repaired or repaired incorrectly, DSBs will lead to chromosomal aberrations and cancers or cells death [123], [124]. These breaks are normally immediately repaired unless the mechanisms for repair recognition or damage processing are faulty such as in some inherited diseases in which there is a genomic instability. Organisms have evolved two major pathways to repair DSBs, homologous recombination (HR) and non-homologous end joining (NHEJ) [124]. HR repairs the broken DNA by utilizing undamaged identical DNA sequence in the genome (homologous chromosome) to serve as a repair template [125]. In NHEJ, DNA break is repaired by recruiting different components to the break site to rejoin the broken ends of a damaged DNA molecule [126]. In HR, the sister chromatid is recruited as a template, therefore it is restricted to the S and G2 phases of the cell cycle. On the other hand, NHEJ does not need a template, hence it is active

throughout all phases of the cell cycle [127]. The balance between these pathways is heavily regulated to maintain its genome stability. The complexity of the break affects the balance between HR and NHEJ and several proteins are also known to play an important role [128].

1.5 Genotoxicity and cytotoxicity of retinoids

The genotoxicity of retinoids has been a controversial subject for several years. Different studies proposed a protective effect of retinoids against cancer. It was demonstrated that some retinoids had reduced sister-chromatid exchanges in human diploid fibroblasts [129]. On the other hand, other studies showed that five other retinoids including 13-cis-retinoic acid caused dose-dependent sister-chromatid exchange in the same cells [130]. In addition, retinol and retinal induced the formation of 8-dihydro-2'-deoxyguanosine, and oxidative agent that caused DNA damage in HL-60 cells but not in hydrogen peroxide (H_2O_2)-resistant HP100 cells [131]. Moreover, supplements with retinol led to DNA single strands breaks, DNA fragmentation and production of 8-oxo-7, 8-dihydro-2'-deoxyguanosine in cultured Sertoli cells [132], [133]. Retinol also induced chromosomal aberrations in human lymphocyte cultures [134]. Furthermore, retinol showed mutagenic action in mouse lymphoma cells exposed to UVA [135].

A study conducted by the alpha-tocopherol, beta-carotene cancer prevention reported that supplementation with β-carotene increased the incidence of lung cancer in smokers [136]– [138], but it was demonstrated in a different study that people with higher serum β-carotene levels have a lower risk of cancer [139]. Later study reported that ATRA and a steroidal analogue EA-4 induced double strand DNA fragmentation in C2C12 mouse cells and HL-60 human acute myeloid leukemia cells [140].

Viability in proliferating cells is defined as the capacity of cells to perform all metabolic processes that are essential to preserve the structural and functional integrity of the cells including proliferation. On the other hand, cytotoxicity is the ability to destroy living cells [141]. Cytotoxicity can be caused by chemical compounds or exposure to other cells like natural killer or T cells [142], [143]. A previous study examined the association between cytotoxicity and DNA strand breaks on primary rat hepatocytes showed a substantial increase in DNA damage at approximately 70% cytotoxicity in the MTT and intracellular ATP assays [144] . A later study, applying the intracellular ATP assay also confirmed this association in rat hepatocytes using 3 hours exposure [145]. Therefore, to avoid false classification of a compound as a genotoxin, it is

necessary to study the viability or cytotoxicity before testing DNA-damaging effects [141]. Indeed, there are not many studies or reviews, which used or suggested a threshold level of cytotoxicity prior to the study of genotoxicity. However, a threshold between 20% and 30% cellular cytotoxicity or cell death is most commonly recommended [146], [147].

1.6 Retinoids and TRPV1, TRPA1 channels

It is well observed that topical application of retinoids often leads to severe local irritation, itching and stinging [148], [149]. Systemically administered retinoids also cause bone pain, and severe headache [148], [150]. Other studies showed that oral or intrathecal application of ATRA induced nociceptive behavioral effects in rodents [151], [152]. In addition, recent studies have reported that retinoids selectively sensitized the capsaicin receptor TRPV1 and produced sensory hypersensitivity [153]. Furthermore, the selective RAR receptor antagonist LE135 activated both TRPA1 and TRPV1 channels and induced pain-related behaviors [154]. In contrast, some antagonists of RAR attenuated retinoid-evoked irritation [152].

Recent studies reported that different members of the TRP channels play important roles in oxidative stress leading to cell injury. TRPV1 is associated with oxidative stress- induced pain and neuronal injury, seen in neuropathy, neurodegenerative diseases, and glaucomatous optic neuropathy [155]. However, it has not been established whether TRP channels are mediators of the cellular responses leading to DNA damage and/or to the painful and inflammatory side effects associated with retinoids. In this thesis, we demonstrate our preliminary study to test this idea.

Novel diphenylacetylene-based ATRA derivatives with a broad range of structural variations were synthesized according to published procedure [156] (Figure 7) in order to increase the stability and reduce susceptibility to oxidation and isomerization. DC360 emits fluorescence when activated by visible light (380-420 nm); DC324 is a DC360 derivative with an expanded structure that disrupt binding to retinoid nuclear receptors and transport proteins, hence, it is a non-active fluorescent retinoid [66]. EC23, DC525, DC540, DC645 and DC712 have increased receptor binding and bioactivity [66], [157], [158]. These synthetic ATRA analogues were reported to cause differentiation in, for example, neuroblastoma cell lines, similar or more strongly than ATRA [159], [160]. They have great potential as therapeutics for a variety of cancers and neurodegenerative diseases including amyotrophic lateral sclerosis [161].

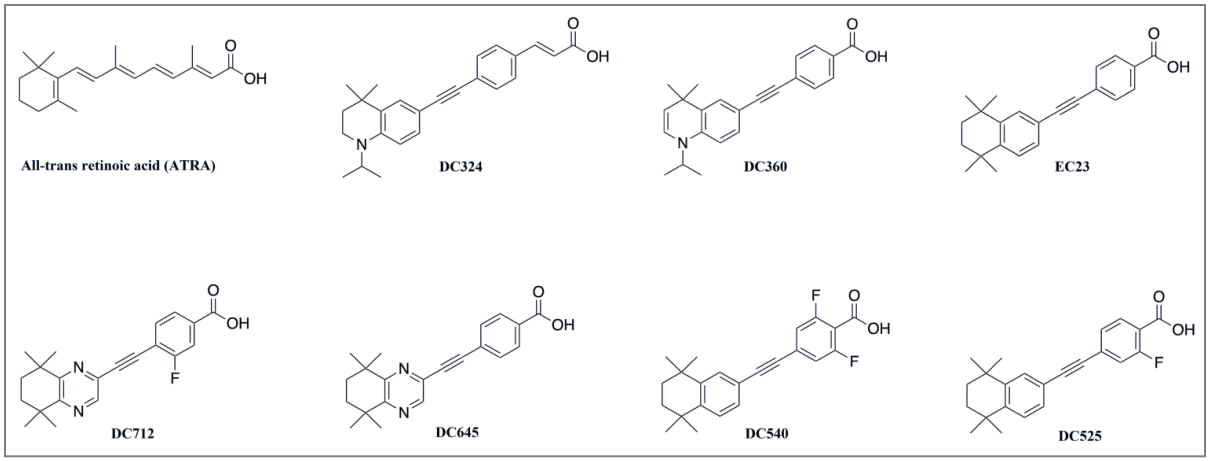

Figure 7. Chemical structures of ATRA and its analogs.

2. Aims of the study

The studies of cytotoxicity and genotoxicity are important parameters for the design and development of novel analogs. Since, there are no data about the potential effect of these compounds on normal cell viability and DNA integrity, the aim of this study was to investigate the effect of novel diphenylacetylene-based retinoids on cell viability and DNA stability on normal CHO cells before proceeding to TRPV1, TRPA1, and pain- related studies which have been characterized and are ongoing at the time this dissertation is written. CHO cells were the cells of choice as they are widely used in comet assay because they are easy to handle and can highly tolerate variations in temperature, oxygen levels and pH.

3. Methods

3.1 Cell culture

The CHO-K1 cell line (ATCC, Virginia, USA) was chosen as fast-dividing cells with subculturing rate of 1:4–1:8 according. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 4 mmol L-glutamine, 10% fetal bovine serum (FBS), 1x penicillin/streptomycin (Thermo Fisher Scientific) and kept at 37 °C in a 5% CO_2 incubator.

3.2 ATP cell viability assay

Damaged and leaky cell membranes characterize the dead cells which cannot be repaired [162], therefore, they can be detected by measuring movement of molecules into or out of cells. Some

marker molecules are typically found in the cytoplasm of viable cells but will move into the culture medium after loss of membrane integrity [163]. They also include the so-called “vital dyes” such as trypan blue and many fluorescent DNA binding dyes. Viable cell membranes are usually not permeable for these dyes, but in dead cells these dyes can pass through the leaky membranes, hence staining them [164]. Different cell-based assays are available and often used for screening of compounds to determine if they have any effects on cell proliferation or show cytotoxic effects that will eventually cause cell death [165]. The most commonly applied technique is the measurement of ATP using firefly luciferase. The concept behind using the ATP as a marker of viable cells relies on the fact that dead cells lose their ability to synthesize ATP and the endogenous ATPases can rapidly deplete the remaining ATP inside the cytoplasm. Recent updates in assay design have led to homogenous protocols with a single reagent addition, which can induce a luminescent signal able to be detected for several hours [166].

Initially, 500μM stock solutions of compounds in absolute ethanol (vehicle) were prepared. CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Mannheim, Germany) was used to detect the number of viable CHO cells in culture. CHO cells were seeded with a density of 5000 cells/ well in a 96-well plate in DMEM supplemented with 10% FBS, 2mM L-glutamine and incubated for 24 hours at 37°C, 5% CO_2. Then, cells were treated with 10 μM and 1 μM ATRA or its analogs and incubated at 37 °C, 5% CO_2 for another 24 hours. When incubation was done, 100 μl CellTiter-Glo reagent was added to each well. The content was mixed by placing the plate onto an orbital shaker for 2 minutes. The plate was then incubated for 10 minutes at room temperature (RT), and cell viability was measured using a plate reader luminometer (EnSpire AlphaLISA, PerkinElmer, Inc, Waltham, MA, USA) as relative light units (RLU) at an integration time of 0.25–1 second per well. Wells. Viability was calculated using the background-corrected luminescence as follows: Viability (%) = RLU of experiment well/RLU of control well × 100 Wells containing only 100 μl cell culture medium or vehicle were considered as negative controls. The viability of treated cells was determined by comparing levels of treated cells to the vehicle-

treated controls. The experiment was performed twice with 6 wells/ compound each time. See Figure 8.

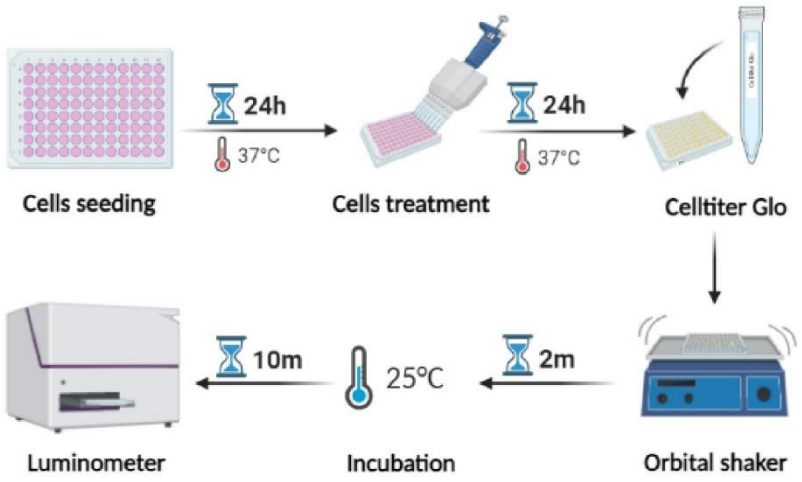

Figure 8. Demonstrative cartoon of ATP-viability assay protocol. This figure was created with Biorender.com.

3.3 Comet assay (Single cell gel electrophoresis)

Comet assay or single cell gel electrophoresis is one of the most common methods for evaluating DNA damage and repair in eukaryotic cells or dissociated tissues [167]. This technique measures DNA breaks by identifying the head of the comet as a mass of undamaged DNA, and the negatively charged fragments that are drawn toward the anode as the tail of the comet [67]. The microgel electrophoresis technique for detecting DNA damage was first developed by Ostling and Johanson in 1984 [168]. Previous studies investigated the nuclear structure by lysing cells with nonionic detergent and high sodium chloride content [169], [170]. This treatment removes cell and nuclear membranes, and almost all histones. The remaining bodies resemble nuclei in size and shape and are known as nucleoids. Nucleoids consist of a nuclear matrix composed of RNA, proteins, and DNA [167]. Extraction of histones disrupts the chromosomal organization of DNA, but the matrix like structure within the nuclear core keeps the DNA attached as a series of supercoiled loops that are conserved if the DNA is intact. When the supercoiling is unwounded by introducing an intercalating agent, the loops extend further towards the anode under electrophoresis forming the comet tail. The number of these loops in the tail (tail's intensity) indicates the number of breaks in DNA suggesting that tail's intensity increases when the DNA damage increases [171].

At first, frosted microscopic slides were coated with 0.8% normal melting point agarose and stored for future use. The cells were treated with the ATRA, DC324, DC360, EC23, DC712, DC645, DC540, DC525 for 24 hours or H_2O_2 for 15 minutes as positive control. Untreated and vehicle-treated wells served as negative controls. The tested concentrations of compounds were chosen to be non-cytotoxic as determined by ATP viability test. The cells were then detached, centrifuged, resuspended, and mixed with 0.7% low melting point agarose at 40 °C. Then, 200µl of the sample was spread onto the pre-coated slide (3 slides/ treatment) and covered with a coverslip. The agarose was allowed to solidify by placing the slides at 4 °C for 5 minutes. Then, the uncovered slides were dipped in an alkaline lysis solution (pH 10) at 4 °C overnight. On the next day, electrophoresis was performed with a cold electrophoresis buffer (pH 13), at 25 V, 300 mA for 30 minutes. The slides were washed for 5 minutes with neutralizing buffer (pH 7.5), then distilled water for 5 minutes, and absolute ethanol for 5 minutes. The slides were air-dried for 15 minutes before staining with 20,000x Eco-safe for 10 minutes, in the dark at RT. Excess stain was washed by immersing the slides in distilled water, and then 2-3 drops of fluoromount solution were added and covered with a coverslip. Samples were photographed using fluorescent microscope at 200x magnification. 50 cells/experiment were selected randomly for the analysis of comet by quantifying the DNA damage as DNA% in the tail. Comet images were analyzed using OpenComet plugin in ImageJ software. All steps are summarized in Figure 9.

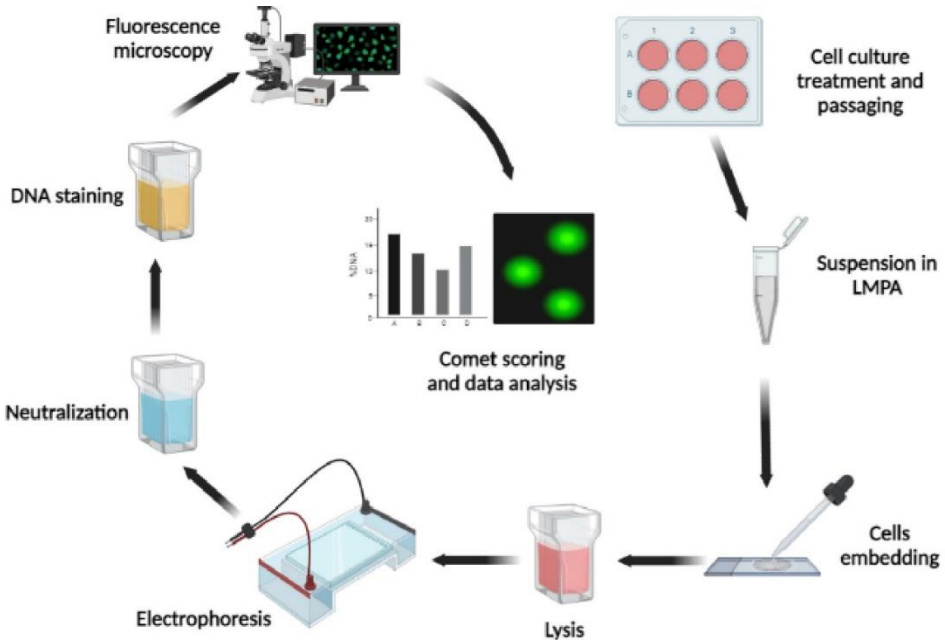

Figure 9. General comet assay procedure. This figure was created with Biorender.com.

3.4 Statistical analysis

All results were subjected to statistical analysis by Graphpad Prism (Version 8.0.1). Using the Shapiro–Wilk test, data were not normally distributed. Therefore, one-way ANOVA along with Kruskal Wallis test followed by Dunn's multiple comparisons test was performed. The results are presented as mean ± SEM at $P < 0.05$ level of significance.

4. Results

4.1 ATRA and its novel synthetic derivatives do not have cytotoxic effect on CHO cells

To select a non-toxic concentration of our compounds, the study of cytotoxicity was performed before the study of genotoxicity. Neither ATRA nor any of the synthetic retinoids showed cytotoxic effect at 1 μM (Figure 10. A). DC324 showed statistically significant decrease in cell viability compared to vehicle control at 10 μM (Figure 10. B). However, it retained more than 70% relative survival that is needed for the comet assay.

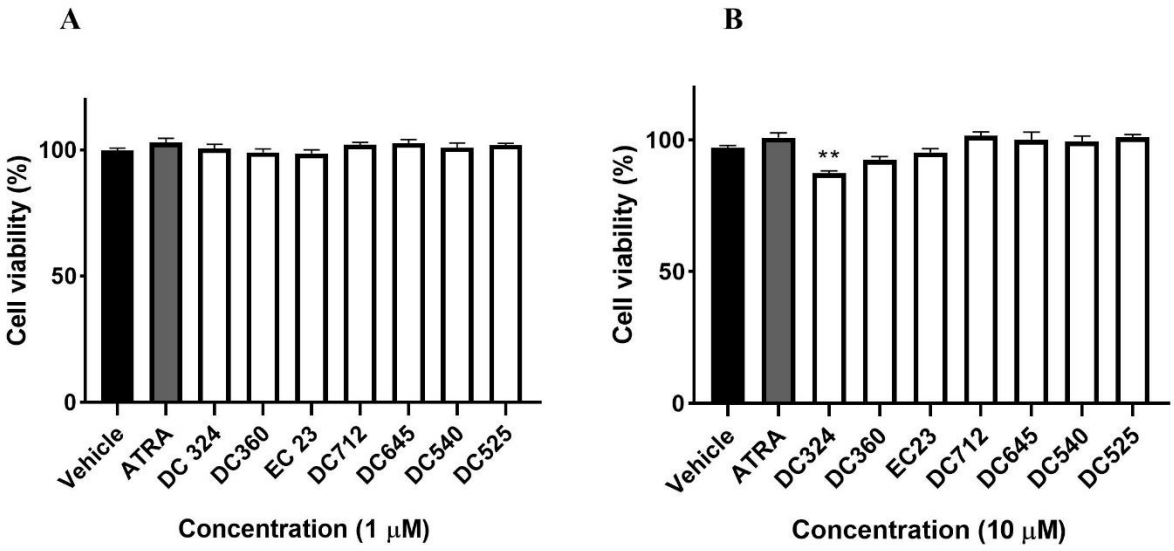

Figure 10. Viability of CHO cell. Cells were exposed to ATRA or its synthetic analogues at concentrations of 1 μM (A) or 10 μM (B) for 24 hours and cell viability was detected by the ATP viability assay as a percentage of the vehicle-treated control. Each column represents the mean ± SEM (** $p < 0.01$ vs. vehicle).

4.2 The synthetic retinoids induce similar or greater DNA damage as ATRA

Representative fluorescence comet images of CHO cells treated with ATRA, DC324, DC360, EC23, DC712, DC645, DC540 and DC525 compared to the vehicle control and H_2O_2 in 10 µM concentration are shown in Figure 11.

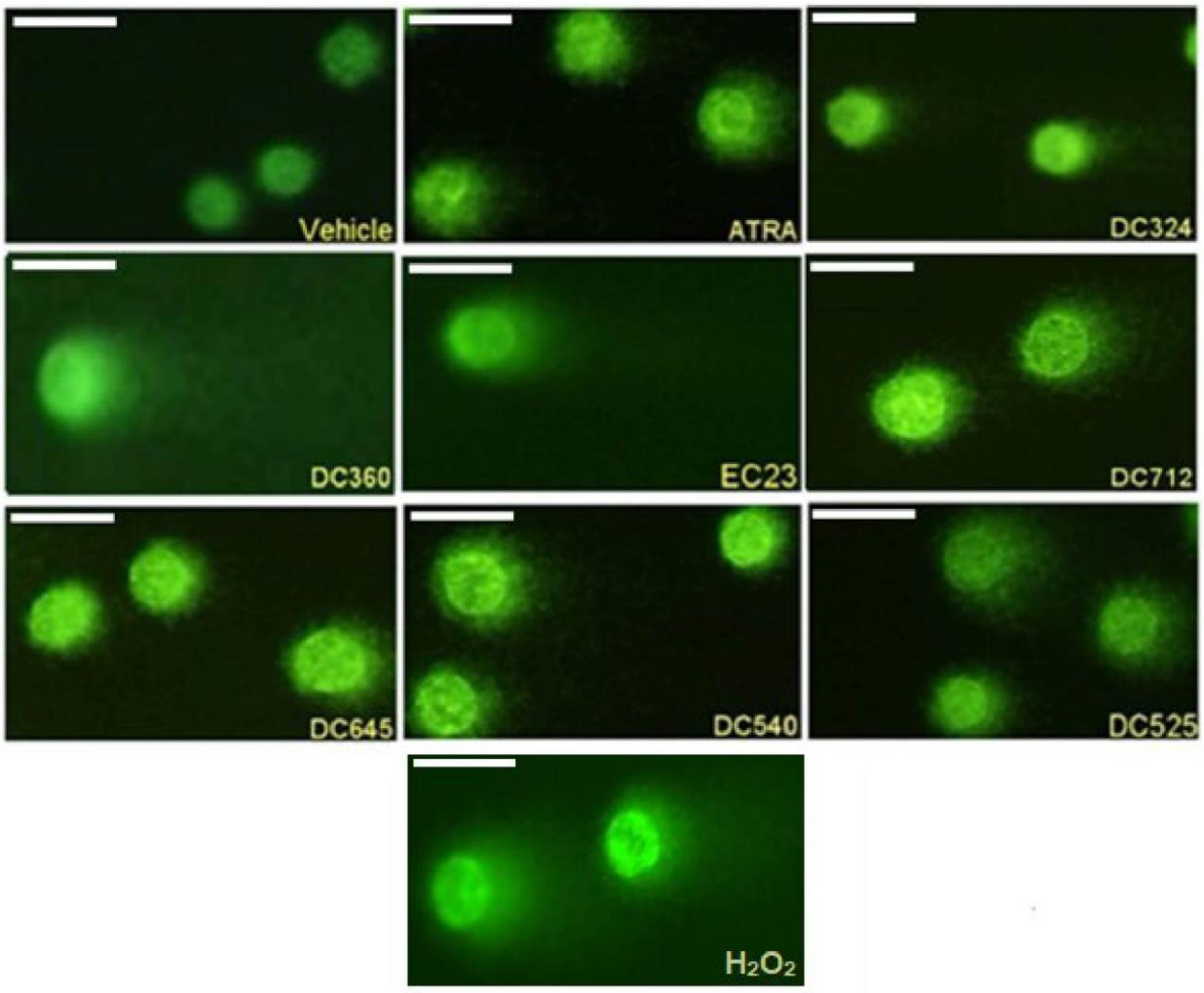

Figure 11. Representative comet images. CHO cells treated for 24 hours with vehicle, 10 µM ATRA, DC324, DC360, EC23, DC712, DC645, DC540, DC525, and H_2O_2 for 15 min. Magnification 200×.

The positive control H_2O_2 significantly increased, by almost double the %tail DNA compared to the vehicle control at 10 µM (Figure 12. A). ATRA significantly increased %tail DNA to a lower extent at 1 and 10 µM but not at lower concentrations (Figure 12. A-D). All tested concentrations

of DC360 significantly increased %tail DNA compared to both vehicle control and ATRA at a similar extent to H_2O_2 (Figure 12. A-D) as well as the 1 μM of DC324 (Figure 12. A).

In addition, we observed significant elevation of % DNA in tail with EC23 at concentrations between 100 nM - 10 μM when compared to both vehicle control and ATRA with no significant increase at 10 nM (Figure 12. A, B, C, D).

DC645, and DC525 elevated % DNA in tail significantly at 10 μM compared to control and ATRA, while DC712, and DC540 increased % DNA significantly compared to control only (Figure 12. A). At 1μM, these 4 compounds showed significant increase compared to vehicle control only (Figure 12. B).

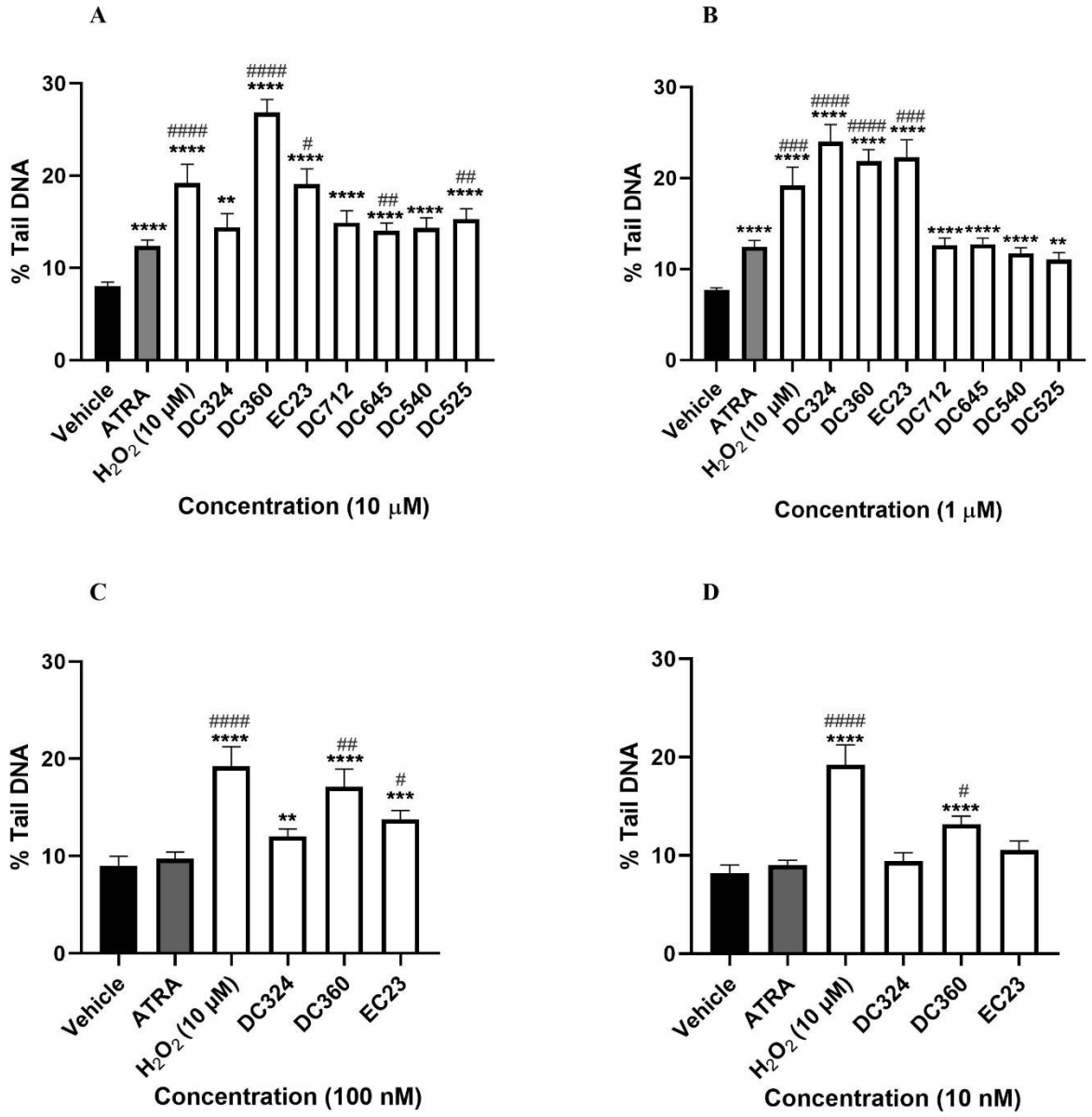

Figure 12. Degree of DNA-damage following retinoid treatments. Columns represent the %tail DNA of CHO cells treated for 24 hours with vehicle, 10 μM H_2O_2 as the positive control, and the retinoid compounds (10 μM (A), 1 μM (B), 100 nM (C), 10 nM (D)). Each column demonstrates the mean ± SEM (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs. vehicle), (# $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$ vs. ATRA).

5. Discussion

We present the first functional data about the effects of seven novel diphenylacetylene-based synthetic retinoic acid analogs on cell viability and DNA stability. It is shown here that these retinoids do not have cytotoxic effects, but cause genotoxicity demonstrated as DNA strand breaks

in CHO cells. DC360 showed the greatest increase of %tail DNA even at the lowest tested concentration, while the other derivatives had DNA damaging effects like that of ATRA.

Previous *in vivo* and *in vitro* studies have demonstrated that retinoids are beneficial in prevention and treatment of cancer [172], [173], but their toxicity has held back their clinical use [174], [175]. It is well recognized that DNA damage is a critical factor in cancer development, where the induction of chromosome breakage is regarded to be genotoxic and potentially carcinogenic [176]. DNA is susceptible to a wide range of endogenous and exogenous alterations that may cause mutations [91]. Genomic DNA can be attacked by chemical or physical mutagens affecting transcription, replication and chromosome segregation [177], which may lead to spontaneous damage such as base modifications, strand breaks and strand cross links [178], [179]. This damage activates specific proteins to stop the cell cycle until the damage is repaired [180]. In case of irreparable damage, the cells undergo permanent cell-cycle arrest, senescence or cell death (apoptosis/necrosis) [181]. On the other hand, if the DNA damage is successfully repaired, apoptosis signaling proteins will not be activated, thus the viability of cells is preserved [182]. Therefore, we believe that our retinoic acid compounds cause repairable DNA damage.

When tested at high concentrations, a non-genotoxic agent may cause DNA fragmentation and cell death, thus cytotoxicity may lead to false positive results in some genotoxicity assays. Therefore, in our study, a non-cytotoxic concentration was chosen to test the direct effect of the compounds on DNA damage. At the tested concentrations (1 μM and 10 μM), ATRA and the synthetic analogues did not significantly decrease cell viability, except for DC324, which caused statistically significant decrease, but the inhibition was only 13%. This is less than the generally accepted guidelines for the comet assay, which recommend a threshold of 25% affected cells as a good starting point to avoid false positive results. Therefore, all compounds could be further investigated in the genotoxicity comet assay. Different viability and cytotoxicity assays depend on measuring the metabolic or enzymatic activity of the cells and are currently widely used in combination with the comet assay. The ATP viability assay is a homogenous, sensitive method used as a valid marker of viable cells because ATP is synthesized by a process called cellular respiration to be used as an energy source in different other processes in cells. This assay can detect as low as 10 cells per well in a rapid and robust way, within 10 minutes to several hours after adding the reagent [166]. In our study, we treated our cells for 24 hours as longer incubation periods are not recommended or appropriate for the comet assay as DNA lesions may be repaired

during the time and applied ATP viability assay to determine the cytotoxicity of our compounds. However, if the aim is to elucidate the mechanism of cytotoxicity, different apoptosis assays can be performed, such as annexin v and caspase assays [183].

Our results regarding the DNA damaging effects of ATRA on CHO cells align with earlier findings in other cell types. For instance, ATRA triggered apoptosis in HL-60 human acute myeloid leukemia cells by typical DNA fragmentation [184], and in two human hepatoma cell lines, HepG2 and Hep3B, as previously assessed by flow cytometry [185]. ATRA and a steroidal analogue EA-4 also induced DNA fragmentation in both HL-60 and C2C12 mouse myocyte cells [186]. In addition, an increase in the level of intracellular ROS and DNA damage was observed when ARPE-19 cells were treated with ATRA after exposure to tert-butyl hydroperoxide [187]. Our study shows that all synthetic ATRA derivatives induced similar DNA damaging effects, but the most hydrophobic compounds, DC324, DC360 and EC23 were even more toxic than ATRA, particularly at higher concentrations. Since these compounds have high log P structures, they may exhibit stronger off-target interactions with other proteins and DNA species which explain this peculiar effect. DC360 caused the strongest genotoxic effect compared to the others, this can be explained by the enamine function of the dihydroquinoline hydrophobic region of this compound which could also be reactive towards cellular components under certain circumstances. DC324, an inactive retinoid that lacks the ability of binding to the retinoid receptors, induced remarkable DNA damage. Thus, these observed genotoxic effects are most likely to be unassociated to the receptor-mediated mechanism. However, further studies are required to elucidate the molecular mechanism of these genotoxic effects.

The comet assay is an efficient, widely used method for genotoxicity and biomonitoring as it is able to quantify the extent of DNA damage at the cellular level [188]. But as any other *in vitro* assay, it has its own limitations. For example, comet assay represents the ratio of the fragmented DNA, hence, to understand the molecular mechanisms in precisely, other assays such as γH2A.X staining and immunoblotting should be performed [189].

Previous studies have proven retinol and β-carotene to have pro-oxidant effects that might lead to oxidative DNA damage and carcinogenesis [190]– [192]. When supplemented at doses higher than the recommended dietary intake, they can generate free radicals that contribute to an increased incidence of different types of cancer, such as esophagus, oral cavity, pharynx, larynx, stomach,

colon and rectum [193]. Upon treatment of primary cultured Sertoli cells with retinol, a stimulation of antioxidant enzyme activities and oxidative damage was observed, which was explained by the increase of iron uptake and storage and the synthesis of highly reactive hydroxyl radicals by the Fenton reaction [132], [194]. These radicals can cause DNA breaks by directly attacking the deoxyribose structure [195]. In addition, retinol changed the function and organization and of rat Sertoli cells chromatin, which lead to alteration in the on- and off-switching of transcriptional-active regions of DNA [196]. Moreover, retinol induced DNA breaks, cell cycle progression, and increased the numbers of proliferative foci in terminally differentiated rat Sertoli cells, and increased DNA fragmentation in Chinese hamster lung fibroblasts [197]. All the mentioned mechanisms might contribute to the observed genotoxic effects of our novel compounds in CHO cells.

In conclusion, this study shows that the novel synthetic diphenylacetylene-based ATRA analogs are not cytotoxic, but they trigger DNA fragmentation and migration due to DNA strand breaks which can lead to genotoxicity and genome instability. Our fluorescent compound, DC360, induces the most pronounced DNA damage. Further studies are needed to identify the underlying molecular mechanisms and elucidate the complex biological activities of these compounds. Based on the presented findings, we believe that these compounds demonstrate a retinoid receptor-independent genotoxicity and that should be considered in later development and applications.

II. Functional expression of TRPA1 ion channels in mouse osteosarcoma K7M2 cells

1. Background

1.1 Osteosarcoma

OS, the most common primary malignant tumor of the skeleton, is a very heterogeneous cancer that is inevitably linked to its microenvironment, which mediates tumor cells survival and growth [198]. Despite the advances in clinical management of OS, the survival rates for patients with metastatic and relapsed disease remain below 20% [198], [199]. Therefore, new agents that induce suppress the growth and survival of OS cells might offer potential therapeutical benefits.

1.2 Etiology of osteosarcoma

OS is believed to be caused by DNA mutations that were either inherited or acquired throughout a person's life. Increased risk has been associated with inherited disorders such as hereditary Li Fraumeni syndrome, Rothmund–Thomson syndrome, and retinoblastoma. Teenage growth spurts and previous exposure to high doses of radiation, particularly at a young age increase the risk for bone cancer [200]. A series of 962 OS identified that skeletally immature patients diagnosed with OS had a higher mean height than a reference standard population [201]. In young patients, OS usually starts in the metaphysis of long bones mostly during puberty, a time of growth spurts and high cellular activity. The peak incidence observed in females is 12 years compared to 16 in males. OS is rarely seen in paediatrics, while 10- 20% elderly patients develop OS as a consequence of Paget's disease [202].

1.3 Management of osteosarcoma

Several advances in imaging, surgical expertise, chemotherapy, and biomedical engineering have recently been applied which resulted in improved OS treatment. Any OS that can be resected should be operated to increase the chance for local control and survival. Neo-adjuvant chemotherapy is very important before surgery, particularly with high grade tumors to target micro-metastases and induces tumor necrosis. The most effective used agents in chemotherapy are high-dose methotrexate, doxorubicin, and cisplatin [203]. Neoadjuvant chemotherapy should be followed with local curative therapy including limb salvage surgery or ablative surgery with amputation and rotationplasty. This is usually followed by a second course of chemotherapy [204]. Bone healing is necessary for the success of limb salvage reconstructions. However, the loss of bone after surgical resection of tumors and compromised wound healing due to the use of cytotoxic

chemotherapy are challenging problems that impair the healing process [205]. Unfortunately, not all OS tumors are operable, some lesions are too large or close to vital structures hindering their complete resection. In these cases, chemotherapy is the first treatment aiming to shrink the tumor enough to become resectable [206]. When the tumor is still unable to be completely removed, different chemo agents could be tried, or radiation therapy is applied to keep it under control and decrease symptoms [207]. Due to all these complications, newer treatment options are needed in many OS cases.

1.4 Osteosarcoma models

A full understanding of OS origin and etiology is complicated by several factors. Such factors include the low overall prevalence of OS compared to other tumor types, the extremely rearranged genome and high genetic instability, the heterogeneity of tumors, and limited human samples. The administration of high-dose chemotherapy during the course of treatment eradicates all viable cancer cells in the resected lesion making it hard to imply it for further studies. Therefore, representative models of OS are highly needed to provide additional samples to study the tumor biology and its cancerous behavior, and to test different therapeutic options in pre-clinical trials [208].

Until today, there is no ideal model, which fully represents the biological, genetic, and clinical features of OS. Different cell lines have been established to study specific genetic changes or investigate the response to different agents that target specific proteins or pathways. The first and most used OS cell lines are those derived from lung metastases of spontaneous OS in BALB/c mice including K12 and K7M2 cell lines. In addition, different human OS cell lines have successfully been established such as such as MG63, SaOS, and 143B. Appropriate cell lines can be selected based on the study interest. Murine models of OS have also been established either by genetic modifications, radiation, or by inoculation of human, murine, or even canine cell lines into mice. The mouse model has been proven to be great for identifying factors involved in OS metastasis and for screening therapies that can inhibit this. Other animal models such as zebrafish or models have recently been used. Each model has its own limitations but establishing an adequate model may help understanding the OS pathophysiology, hence improving the development of therapies in the future [208], [209]

Based on the studies showing TRPV1 and TRPA1 channels implication in multiple events controlling cellular fate and cancer progression, thus the objectives of this study are to investigate their functional expression and examine whether their activation induces cell death in OS K7M2 cells. These findings may have therapeutic implications for frequently fatal OS.

1.5 TRPA1 channel in pain and cancer

TRPA1 is the only member of the ankyrin subfamily. It is highly permeable to Ca^{2+} and is activated by exogenous agonists, including cinnamaldehyde (in cinnamon), allyl isothiocyanate (AITC, in mustard oil and wasabi), and allicin (in garlic), and endogenous substances produced at sites of inflammation or tissue injury, such as reactive oxygen, nitrogen and carbonyl species [210].

The first study to demonstrate that activation of TRPA1 is associated with a painful sensation was in 2004 in both *in-vitro* and *in-vivo* models [211]. Several studies have later showed similar results, for example, TRPA1 has been found to mediate inflammatory mechanical hyperalgesia as well as cold hyperalgesia under inflammatory conditions [212], [213] . In addition, TRPA1 modulated inflammation and pruritogen responses in allergic contact dermatitis. Furthermore, TRPA1 has reported to be involved in mechanical hyperalgesia and allodynia of neuropathic pain in diabetic patients or in patients receiving chemotherapeutics, which proposed to be through the synthesis of ROS and nitrogen species [214], [215]. TRPA1 also contribute to the onset of oral cancer pain since these tumors can release lipids that activate can activate both TRPA1 and TRPV1 [216]. In a mouse melanoma model, cold and mechanical allodynia and thigmotaxis behavior were observed 14 days after tumor cell inoculation. These behaviors were attenuated by administration of TRPA1 antagonists (HC-030031 or A-967079) and administration of a TRPA1 antisense oligonucleotide [217].

TRPA1 is expressed in nociceptive neurons and is also expressed in non-neuronal cells, like in lung epithelial fibroblasts [218]. Little is known about TRPA1 functions in malignancies and data linked to this topic is controversial. It was shown that the activation of TRPA1 channels in cancer cells increases Ca^{2+} influx, cell migration, and tumor cell invasion [219], for example, TRPA1 has been indicated for having a protective mechanism in lung cancer progression [220]. In the stromal cells of human prostate cancer, the antibacterial agent, triclosan has been reported to cause TRPA1 activation inducing Ca^{2+} influx, which led to vascular endothelial growth factor secretion and increased cell growth [221]. Data from The Cancer Genome Atlas program shows the association

between high expression of the *TRPA1* gene and improved survival in intrahepatic bile duct, bladder, and liver cancers. However, a confirmed analysis of the possible role of TRPA1 as a diagnostic marker was not completed [222]. *TRPA1* mRNA was significantly expressed in small cell lung cancer cell (SCLC) lines and in SCLC patients when compared to non-SCLC samples or non-malignant lung tissue and activation of TRPA1 by AITC prevented apoptosis and promoted survival [223]. On the other hand, TRPA1 activation by increased mitochondrial ROS production induced Ca^{2+} influx in glioblastoma cells leading to cell death and apoptosis [218], [224]. It was also demonstrated that TRPA1 inhibits pancreatic ductal adenocarcinoma Panc-1 cells migration through both channel dependent and independent pathways [222]. Immunohistochemistry of nasopharyngeal carcinoma patients revealed upregulation of *TRPA1*, which was negatively associated with tumor recurrence and distal metastasis-free survivals [225]. Despite recent studies aiming to clarify the role of TRP channels in progression and metastasis of several types of cancer, little is known about the expression of *TRPA1* in OS. Therefore, our study of *Trpa1* expression in mouse OS K7M2 cells provides new insights on the biology of this tumor.

1.6 TRPV1 channel in pain and cancer

TRPV1 is a non-selective cation channel that belongs to the transient receptor potential vanilloid subfamily which is also named capsaicin (the main pungent component in "hot" chili pepper) receptor and vanilloid receptor 1. This channel can be activated by various physical and chemical stimuli, such as temperatures over 43°C, acidic extracellular conditions (pH <6), and vanilloids [11]. When activated, TRPV1 induces the cellular influx of Ca^{2+} and Na^{+} ions that in excess, leads to cell death [226]. TRPV1 is a promising therapeutic target in different human diseases. Therefore, several agonists and antagonists have been identified and TRPV1 expression has been investigated in health and diseases.

TRPV1 is a polymodal channel sensitive to different thermo-mechanical and chemical stimuli and have a complex role in hyperalgesia and neurogenic inflammation [227]. Due to its high expression in nociceptors, the role of TRPV1 in neuropathic and inflammatory pain had received intense interest among the TRP channels. In vitro studies using human embryonic kidney HEK293 cells transfected with rat TRPV1 have reported TRPV1 activation by different inflammatory mediators including prostaglandin E2 and prostaglandin I2, bradykinin, nerve growth factor and the chemokine CCL3 [228]. TRPV1 antagonists have attenuated thermal hyperalgesia in rat

inflammatory model [229] and similar results have been found in TRPV1 knock out mice model [230]. On the other hand, TRPV1 plays a pronociceptive role in neuropathic pain following nerve injury, chemotherapy, viral infection, or metabolic disorders such as diabetes [231]. In addition, TRPV1 was shown to be activated by ethanol, a well-known trigger of migraine [232]. Chronic pain is a common complication during cancer progression, thus the role of TRPV1 in cancer pain, particularly cancer-induced bone pain (CIBP) has been widely investigated. For example, repeated administration of the TRPV1 antagonist, JNJ-17203212, decreased the ongoing and movement-evoked nocifensive behaviours in different stages of cancer and suppressed spinal c-Fos expression in CIBP mouse model. Furthermore, selective blockade of TRPV1 as well as disruption of the *Trpv1* gene attenuated bone cancer pain in mouse model [233], [38]

It was also proven that TRPV1 level of expression correlates with tumor grading. Previous studies reported that TRPV1 expression is higher in human primary brain cancers than in tumor-free brains [234]. Furthermore, TRPV1 expression was significantly elevated in chronic pancreatitis and human pancreatic cancer compared to a normal pancreas [235]. Other studies have examined the effects of TRPV1 agonists or antagonists on cancer cell proliferation, although some of these compounds regulate proliferation in TRPV1-independent manner as they showed alteration in non-TRPV1 expressing cells. There are numerous reports regarding the ability of capsaicin to induce cell death in urothelial cancer and glioma cells through TRPV1-dependent activation of excessive Ca^{2+} influx. This generates mitochondrial membrane depolarization, alteration in membrane structure and function, and mediates apoptosis [236], [237]. In addition, capsaicin demonstrated strong anti-tumor activity in human OS MG63 cells by activating both TRPV1-dependent (activation of c-Jun N-terminal kinase, mitochondrial dysfunction, overproduction of ROS) and TRPV1-independent (AMP activated protein kinase-p53) signaling pathways leading to cell death [238]. Capsazepine, a synthetic TRPV1 antagonist, and resinferatoxin an ultrapotent plant-derived capsaicin analogue, TRPV1 agonist, inhibited OS induced hyperalgesia in a mouse model of thermal hyperalgesia suggesting a TRPV1-dependent mechanism. In addition, TRPV1 was shown to be expressed in sensory neurons that innervate the mouse femur, and the administration of a TRPV1 antagonist, or suppression of the TRPV1 gene reduced the nocifensive behaviors [29]. The results indicate that TRPV1 channel expression, activation and the subsequent Ca^{2+} signaling play an essential role in cancer cell proliferation and

survival and that additional evaluation of TRPV1 as a pharmaceutical target for the reduction of bone cancer pain will prove beneficial.

2. Aims of the study

Despite the several studies indicating that both TRPV1 and TRPA1 channels play a prominent role in tumor progression. No studies to date have identified the functional expression and/or intracellular localization of TRPA1 in OS cancer. Although TRPV1 has been implicated in the regulation of cancer cell proliferation, migration and invasion, there is a paucity of information regarding functional expression and localization of TRPV1 in OS.

The emerging role of TRPA1 and TRPV1 in cancer metastasis, the tendency of tumor cells to metastasize to the surrounding or distant microenvironment, the colocalization of TRPA1 and TRPV1 in both neuronal and non-neuronal cells and that they may cross-regulate each other's function, prompted us to study the functional expression of both TRPA1 and TRPV1 and the effect of their modulation on the survival of OS cells, thus our aims are:

(I) Characterizing the gene expression of *TRPA1/Trpa1*, *TRPV1/Trpv1* mRNA in human and mouse OS tissues by RNAscope as well as in OS K7M2 cells by RNAscope in situ hybridization and PCR gel electrophoresis.

(II) Investigating radioactive $_{45}Ca^{2+}$ uptake in response to TRPA1 and TRPV1 agonists in K7M2 cells.

(III) Investing the effect of TRPA1 and TRPV1 agonists on OS cell viability by ATP-based luminescent assay.

3. Methods

3.1 Experimental animals

9–10-week-old male BALB/c mice were housed in a temperature and humidity 12-hour controlled light-dark cycle at the Laboratory Animal House of the Department of Pharmacology and Pharmacotherapy, University of Pécs provided ad libidum with standard rodent chow and tap water. All procedures were designed and conducted in full accordance with the European legislation (directive 2010/63/EU) and the Hungarian Government regulation (40/2013., II. 14.), in reference to the protection of animals used for scientific purposes. The study was approved by the Animal Welfare Committee of the University of Pécs, and the National Scientific Ethical

Committee on Animal Experimentation of Hungary (BA02/2000-23/2016). During the experiment, all efforts were provided to reduce the number of animals used and the suffering.

For the intratibial injection of the mouse osteosarcoma K7M2 cell, mice were deeply anesthetized by intraperitoneal injection of Na- Pentobarbital (Euthanimal, Alfasan Netherland, BV). Then one of the hind limbs was shaved and disinfected, after which, a small incision was made on the anterolateral surface under an operating microscope. The tibia was sharply dissected proximally until the metaphyseal flair was identified, where a small hole was created with a 27-gauge needle. The needle was approximately 45° angled in the sagittal plane and advanced to penetrate the cortex, then it was gently twisted to create a cortical window but leave the posterior cortex intact to avoid fracture. After that, 500.000 K7M2 cells in 10 µl phosphate buffered saline (PBS) were injected into the defect with a Hamilton-pipette. The muscle was pulled back over the bone and the skin was closed in a running fashion using a 4-0 vicryl suture. Control animals received a similar treatment but instead of K7M2 cell. sterile saline was injected into the bone. Animals were later awakened and recovered in the usual fashion.

3.2 Human samples

The human samples were taken during biopsy of patients with suspected OS. For confirmation of OS, histopathological and then immunohistochemical experiments were performed. All procedures were approved by the Regional and Institutional Committee of Science and Research Ethics of the University of Pécs (license No. 9624 – PTE 2023).

3.3 Cell line

The mouse OS K7M2 cell line was kindly provided by Krisztina Buzas, Szeged Biological Research Centre. They were maintained in DMEM (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 2 mM L-glutamine, 10% FBS, (Thermo Fisher Scientific) and kept at 37 °C in a 5% CO_2 incubator.

3.4 RNA isolation and PCR gel electrophoresis

K7M2 cell line were homogenized in 1 mL TRIzol-reagent and RNA contents were isolated using Direct-zol™ RNA MicroPrep (Zymo Research, Irvine, CA, USA), according to the manufacturer's instructions. The amount and purity of RNA were obtained by Jenway™ Genova Nano Micro-volume Spectrophotometer (Fisher Scientific, UK). Samples were then treated with

1U DNase I enzyme to eliminate any contemning genomic DNA. 500 ng RNA was used to synthesize cDNA using Applied Biosystems™ High-Capacity Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA).

PCR was performed in a 96-well block using QuantStudio™ 5 system (Life Technologies Magyarország Ltd., Budapest, Hungary). Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) was selected as a reference gene. A reaction volume of 10 µL containing 1x SensiFAST™ Probe Lo-ROX mix (Meridiane Bioscience, Memphis, TN, USA), 20 ng cDNA, and 400 nM probe primer mix (forward and reverse). FAM conjugated TaqMan™ Gene Expression Assays (Thermo Scientific, Waltham, MA, USA) were used to amplify the target loci: *Gapdh*: Mm99999915_g1, *Trpa1*: Mm01227437_m1, *Trpv1*: Mm01246302_m1 , The K7M2 PCR products were electrophoresed on a 2% agarose gel containing 0.01% ethidium bromide at 70 V for 40 minutes and visualized by Molecular Imager BioRad Gel Doc XR+ (BioRad Laboratories) with Image Lab 6.0.1 build 34 software.

3.5 Tissue collection and sample preparation for RNAscope study

Control and tumor injected mice were deeply anesthetized with Euthasol, 14 days after the surgery. Transcardially perfusion was performed by 20 mL of ice-cold 0.1 M PBS, followed by 150 ml 4% paraformaldehyde solution in Milloning buffer (pH= 7.4). The operated hind limb was removed and post-fixed for 72 hours. After decalcification (Morse's solution for two days), samples were paraffin embedded and sliced into 3 □m sections. Human samples were post-fixed for longer time (5 days) and then treated in the same way.

3.6 Cytospin slide preparation for RNAscope

K7M2 cells were centrifuged at RT at 1000x g for 5 minutes. Supernatants were removed and cells were resuspended with media to adjust the cell density to $1x10^6$ cells per ml. Adhesive slides, filter cards, and sample chambers were assembled in the cytospin centrifug and 100 µl of sample was pipetted into each of the sample chambers. Slides were centrifuged at 1000x g for 5 minutes to produce a monolayer on the slide [239]. RNAscope Technical Note for Cultured Adherent cells protocol (Advanced Cell Diagnostics, Newark, CA, ACD, technical note 320538) was used for fixation, post-fixation, wash, and storage steps. After the cytospin, samples were air dried 30 minutes and then fixed using 10% neutral buffered formalin (Sigma) for 30 minutes at RT. Cells

were washed 3 times with 1x PBS and dehydrated in 50%, 70% and 100% ethanol and stored at -20 in preparation for RNAscope staining.

3.7 RNAscope

The RNAscope Multiplex Fluorescent Reagent Kit v2 (ACD, Hayward, CA, USA) was used according to the manufacturer's protocol. K7M2 slides were rehydrated in 70% and 50% ethanol and treated with Protease III, while human and mouse sections were deparaffinized, H_2O_2-blocked, boiled and pre-treated with Protease Plus.

Samples were subsequently hybridized with probes specific to human and mouse tissues and mouse cell lines: *TRPA1/Trpa1* (ACD, Cat. No. ,837411-C2), *TRPV1/Trpv1* (ACD, Cat. No., 415381, ezrin (ACD, Cat. No., 535811) mRNA along with human or mouse 3-plex positive control probes specific to POLR2A mRNA (fluorescein), PPIB mRNA (cyanine 3, Cy3) and UBC mRNA (cyanine 5, Cy5) and 3-plex negative control probes (ACD; Cat. No. 320871) specific to bacterial dabP mRNA. Then, sequential signal amplification and channel development was performed. counterstaining of the nucleus with 4′,6-diamidino-2-phenylindole (DAPI) was then conducted and ProLong Diamond Antifade Mountant was added to slides for confocal imaging.

Fluorescent images were taken with a Nikon Eclipse Ti2-E confocal microscope with 20 x and 60x objectives. Blue, green, red, and white virtual colors were selected to depict fluorescent signals of DAPI (nuclear counterstain), Cy3 (*TRPA1*/*Trpa1* mRNA), Fluorescein (*TRPV1*/*Trpv1* mRNA), Cy5 (ezrin mRNA) respectively. DAPI was excited at 405 nm, Cy3 at 550 nm, Fluorescein at 488 nm, and Cy5 at 650 nm. Z-projection (12-15 stacks/image, 2 µm-interval were chosen for K7M2 cells. Brightness/contrast were processed using (Fiji, 1.53c, NIH, USA).

3.8 Radioactive $^{45}Ca^{2+}$ uptake experiments on K7M2 cells

For receptor selectivity experiments, K7M2 cells were investigated in response to AITC (10 µM, 100 µM, 200) as well as capsaicin (100 nM, 1 µM) (Sigma-Aldrich Ltd, Hungary). these responses were subjected to antagonist treatments by 10 µM HC-030031 (Tocris Bioscience, Cat. No. 2896) or capsazepine (Sigma-Aldrich Ltd, Hungary), respectively. Cells were seeded in 15 µL cell culture medium onto Microwell Minitrays (Merck KGaA, Darmstadt, Germany) and incubated overnight at 37 °C, 5% CO_2 incubator. The next day, cells were washed and incubated in 10 µL Ca^{2+} free Hank's solution (pH 7.4) containing AITC (10 µM, 100 µM, 200) or capsaicin (100 nM,

and 1 µM) and 200 µCi/mL $_{45}Ca^{2+}$ isotope (1.3 Ci/mmol, Ammersham) for 1 minute at RT. Then cells were washed with extracellular solution and the remaining buffer was evaporated, the retained isotope was collected in 15 µL of 0.1% SDS and the radioactivity was measured in 2 mL scintillation liquid in a Packard Tri-Carb 2800 TR scintillation counter. $_{45}Ca^{2+}$ isotope-retention is expressed in count per minute (CPM).

3.9 ATP cell viability assay

Cells were treated with AITC, or capsaicin dissolved in dimethyl sulfoxide (DMSO) using the following concentrations (5 µM, 10 µM, 20 µM, 50 µM, 100 µM, and 200 µM). DMSO concentration of the solvent treated control cells were adjusted to similar concentrations as the concentration of the treatments (1%, 0.5%, 0.2%, 0.1% and 0.05%). For the pre-treatment, cells were treated separately with 10 µM HC-030031 and capsazepine for 10 minutes prior the addition of the agonists AITC and capsaicin, respectively. Cell viability was assessed using a CellTiter-Glo® Luminescent Cell Viability Assay (CTG, Promega, Madison, WI, USA) mixture as recommended by the manufacturer as described previously on page 19.

Cells were seeded in 96-well tissue culture plates at a density of 5000 cells/ well in 100 µl media and cultured for 24 hours. On the following day, AITC and capsaicin were added at the above-mentioned concentrations and cells were incubated for 48 hours. When the incubation was done, the plate and its contents were balanced at RT for approximately 30 minutes. Promega CellTiter-Glo® Reagent was added in a volume equal to the cell culture medium present in each well. ATP-based luminometric measurement from the metabolically active cells in the culture were determined as previously described.

3.10 Statistical analysis

Statistical analyses were performed using GraphPad Prism 8 software. The distribution of the data was examined and passed the Kolmogorov–Smirnov normality test, followed by one-way ANOVA and Dunnett's post hoc test. In all cases, $p < 0.05$ was considered as statistically significant.

4. Results

4.1 *TRPA1/Trpa1* and *TRPV1/Trpv1* mRNAs are expressed in human and mouse osteosarcoma tissues

RNAscope showed both *TRPA1* and *TRPV1* mRNA expression approximately at the same level in two different human OS samples, characterized by osteoid mass with hematoxylin-eosin (H&E) staining (Figure 13. A, D). The mRNA transcripts of both channels were observed on ezrin-positive cells demonstrating that they are OS cells (Figure 13. B, C, E, F).

In addition, the mouse OS samples, seen as similar osteoid mass (Figure 14. C), the same level of expression was observed for both *Trpa1* and *Trpv1* on tumor cells (Figure 14. D, E). Whereas, in control, healthy H&E-stained mouse bone tissue, few transcripts corresponding to both receptors can be seen (Figure 14. A, B).

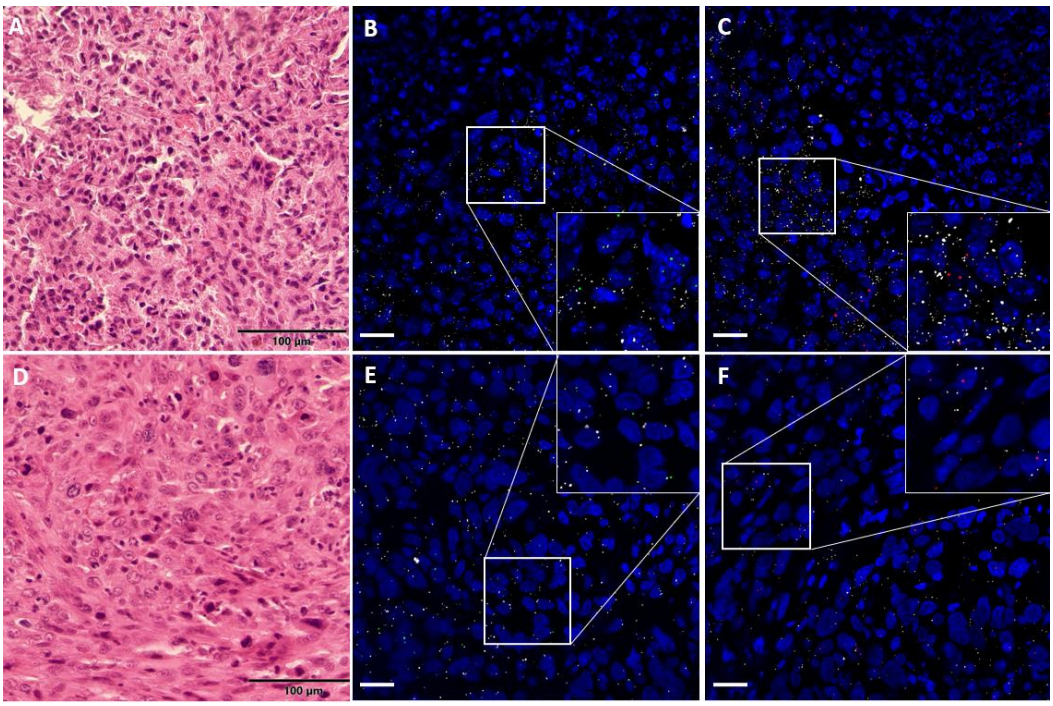

Figure 13. Representative images of *TRPA1* and *TRPV1* expression on OS cells in human tissue. H&E staining of the tissue (A, D). *TRPA1* mRNA (green), *TPRV1* mRNA (red) and ezrin mRNA (white) are visualized by RNAscope. Sections are counterstained with DAPI (blue) for nuclei (B, C, E, F). Scale bars: 25 mm

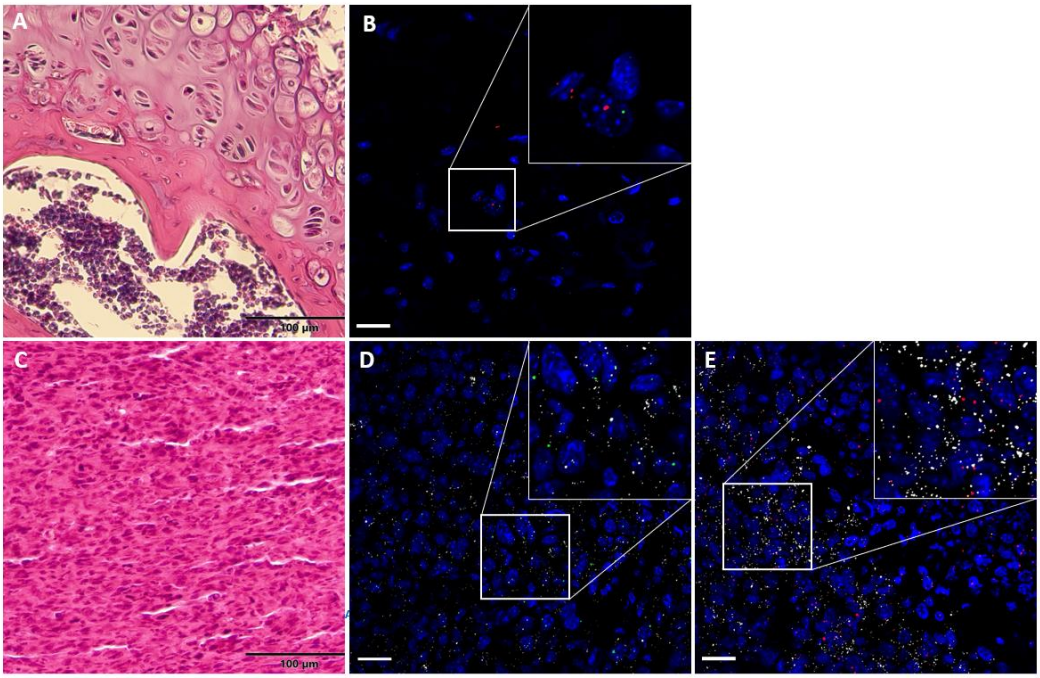

Figure 14. Representative images of *Trpa1* and *Trpv1* expression in mouse tissue. H&E staining of the control (A) and OS samples (C). *Trpa1* mRNA (green) and *Trpv1* mRNA (red) are detected in control (B) and in tumor tissue (D, E). OS cells are identified by ezrin mRNA (white). Sections are counterstained with DAPI (blue) for nuclei. Scale bars: 25 mm

4.2 *Trpa1* mRNA is expressed in osteosarcoma K7M2 cell line

PCR gel electrophoresis showed *Trpa1* mRNA expression with very low *Trpv1* mRNA expression in the K7M2 cell line as presented in (Figure 15. A). In agreement with the gel electrophoresis results, confocal laser scanning microscopy and qualitative morphological evaluation revealed that *Trpa1* mRNA is expressed in the OS K7M2 cell samples, but *Trpv1* mRNA was not detected (Figure 15. B)

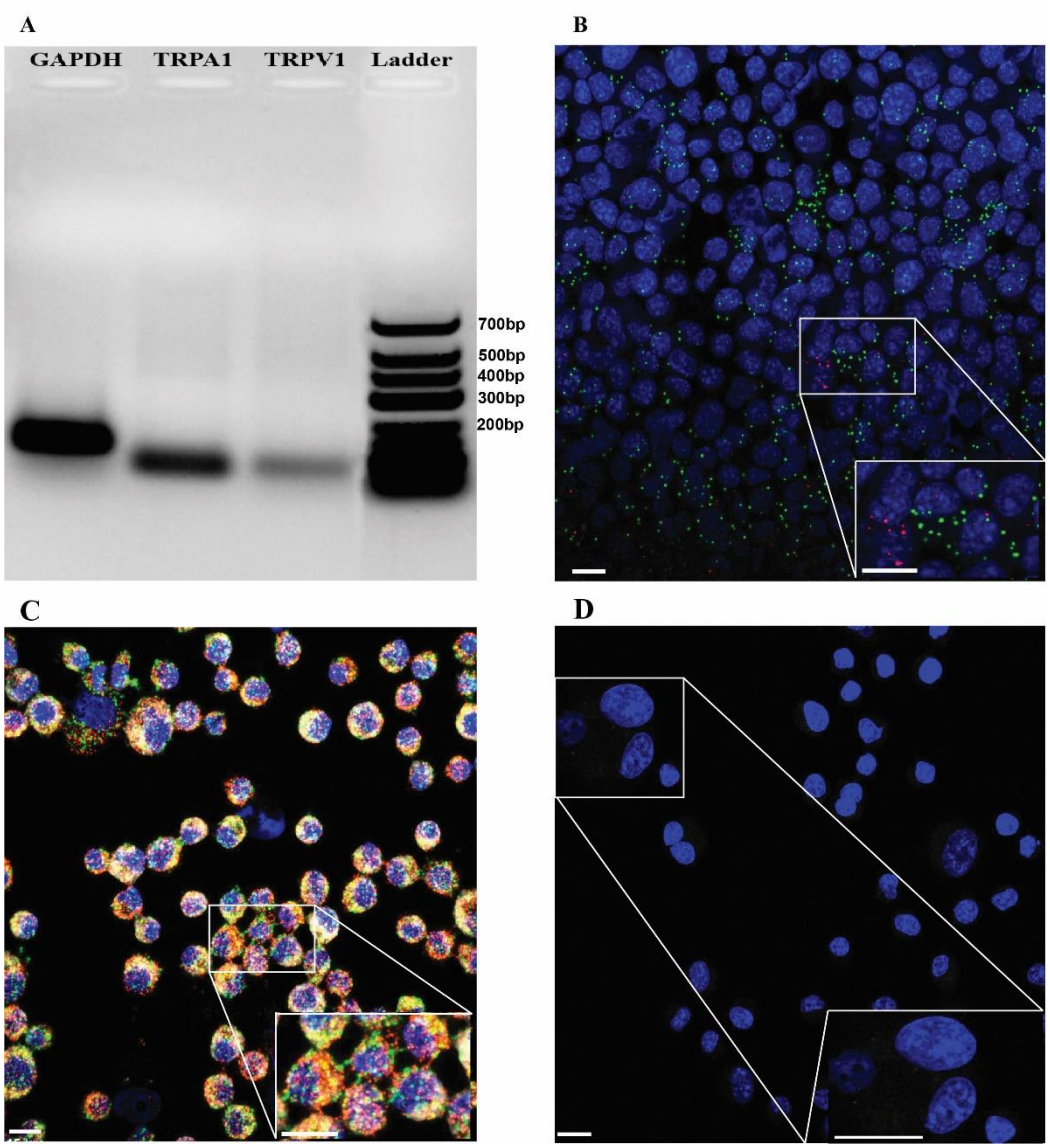

Figure 15. *Trpa1* and *Trpv1* mRNA expression in OS K7M2 cells (A) gel electrophoresis (B) RNAscope (C, D) Positive and negative controls. *Trpa1* mRNA (green) and *Trpv1* mRNA (red) RNAscope were depicted and counterstained with DAPI (blue) for nuclei. Scale bar: 15 µM

4.3 The TRPA1 agonist AITC induces radioactive $_{45}Ca^{2+}$ uptake in K7M2 Cells

TRPA1 agonist AITC (10, 100, and 200 µM) showed concentration-dependent, 400 ± 151, 470 ± 71, and 1037 ± 220 CPM $_{45}Ca^{2+}$ influx into K7M2 cells, respectively (Figure 16. A). The TRPV1 agonist capsaicin at 100 nm and 1 µM did not induce $_{45}Ca^{2+}$ uptake (Figure 16. B). 10 µM of the TRPA1 antagonist, HC-030031, significantly abolished the AITC-induced $_{45}Ca^{2+}$ uptake (255 ± 105 CPM, Figure 16 A).

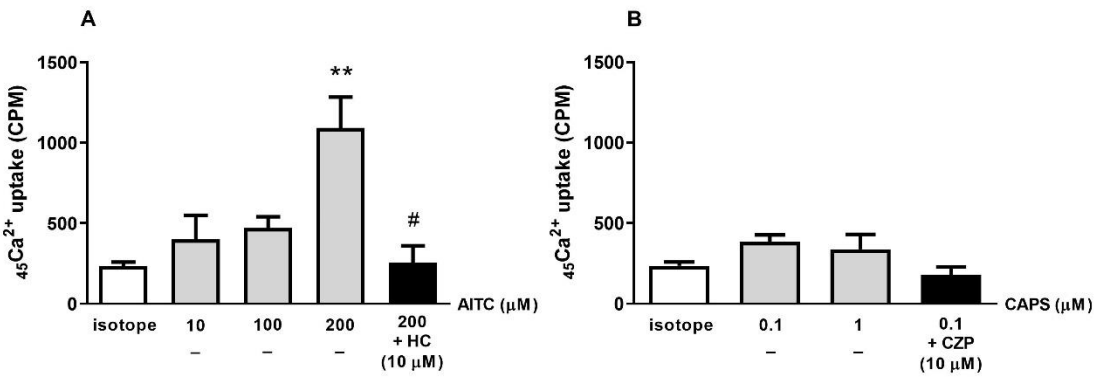

Figure 16. Effect of AITC and capsaicin on $_{45}Ca^{2+}$ uptake (CPM) in K7M2 cells. AITC 200 μM Induced significant $_{45}Ca^{2+}$ uptake (A). Capsaicin showed no significant change in $_{45}Ca^{2+}$ uptake (B) AITC and capsaicin response were blocked by 10 μM HC-030031 and capsazepine, respectively (A, B). Each column represents the mean ± SEM. ** $p < 0.01$, * $p < 0.05$ (vs. control, one-way ANOVA, Dunnett's post hoc test); # $p < 0.05$ (vs. 200 μM AITC, one-way ANOVA, Dunnett's post hoc test). HC: HC-030031, CAPS: Capsaicin, CZP: Capsazepine.

4.4 AITC concentration-dependently reduces osteosarcoma K7M2 cell viability

Incubation of K7M2 cells with five different concentrations of AITC for 48 hours resulted in a significant concentration-dependent cell viability decrease ranging from 74.76% ± 2.767 – 0.2% ± 0.045 at 5 - 200 μM, respectively (Figure 17. A) with EC_{50} of 22 μM ± 1.08 (Figure 17. E). Capsaicin treatment for 48 hours led to significant cell death (viability 79.42% ± 3.731, 65.29% ± 3.515, 45.55% ± 2.188, and 12.46 ± 0.324) at 20, 50,100, and 200 μM, respectively compared to vehicle control. Meanwhile, capsaicin induced non-significant 91.04% ± 3.091 - 92.26% ± 3.386 cell viability at 10 and 5 μM (Figure 17. C). The EC_{50} of capsaicin was 74 μM ± 1.07 (Figure 17. E). In the case of solvent control cells, the vehicle DMSO also affected the cell viability at its highest concentrations (Figure 17. A, C), therefore, our results are expressed in comparison to vehicle control.

10 μm of the TRPA1 antagonist HC-030031 alone did not induced significant decrease in cell viability (Figure 17. A), however 10 minute -pretreatment of cells with HC-030031 caused statistically significant reverse of the AITC-induced decrease in cell viability (Figure 17. B). However, the TRPV1 antagonist, capsazepine exerted slightly significant decrease of viability at 10 μM (Figure 17. C) with no capsaicin-antagonizing effect with 10 minutes pretreatment (Figure 17. D).

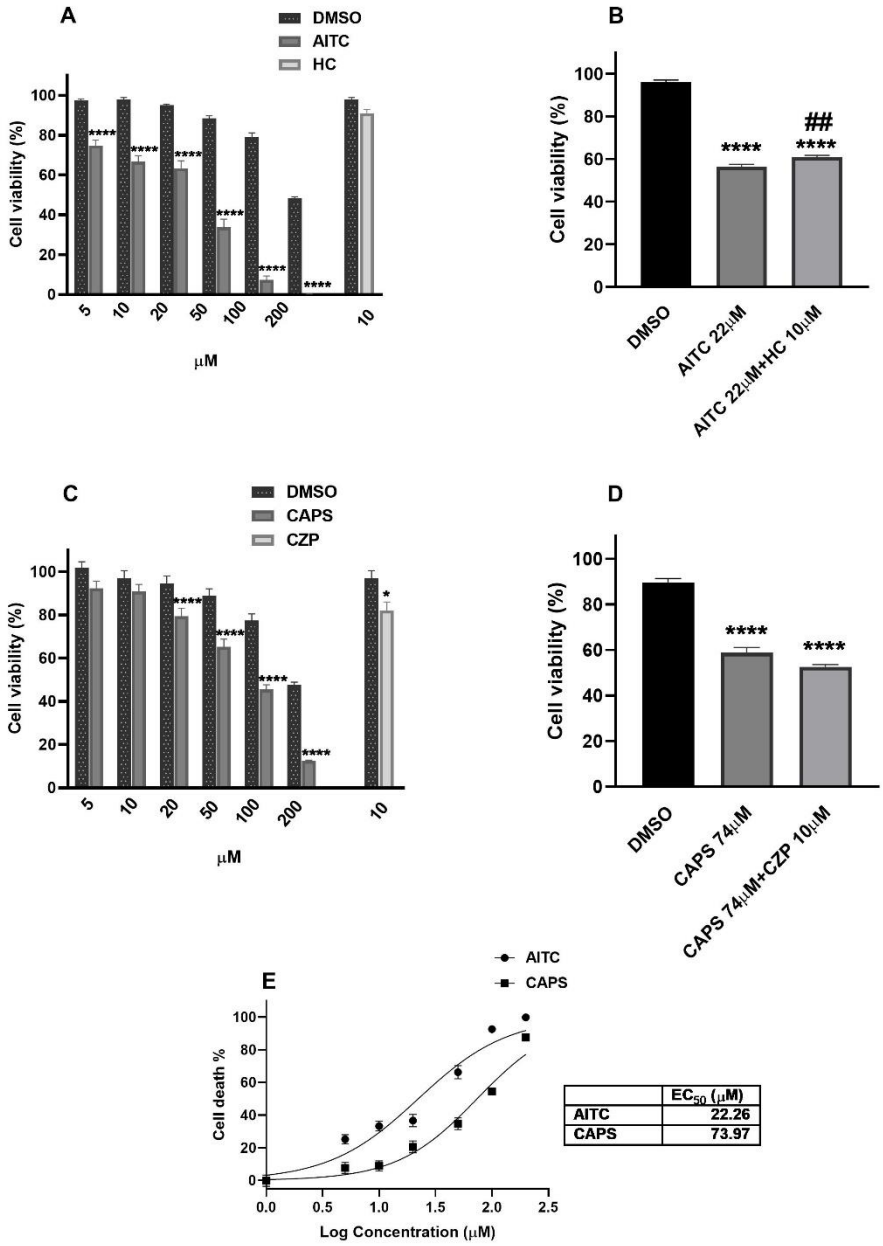

Figure 17. Effect of AITC and capsaicin on the viability of K7M2 cells. K7M2 cells were treated with AITC or capsaicin at 5 μM, 10 μM, 20 μM, 50 μM, 100 μM, 200 μM. (A, C. E) Both treatments showed concentration-dependent reduction in viability of K7M2. The TRPA1 antagonist HC-030031 did not reduce K7M2 cell viability at 10 μM (A). The TRPV1 antagonist capsazepine induced slightly significant reduction in K7M2 cell viability (C). Pretreatment with The TRPA1 antagonist HC-030031 blocks AITC effect (B). Pretreatment with the TRPV1 antagonist capsazepine has no effect on capsaicin-induced cell death (D). All data are expressed in mean ± SEM. **** $p < 0.0001$, ** $p < 0.01$, * $p < 0.05$ (vs. DMSO, one-way ANOVA, Dunnett's post hoc test), ## < 0.01 (vs. 22 μM AITC, one-way ANOVA, Dunnett's post hoc test). HC: HC-030031, CAPS: Capsaicin. CZP: Capsazepine.

5. Discussion

This study provides the first evidence for *Trpa1* and *Trpv1* mRNA expression and TRPA1 and TRPV1 functionality in OS cell line. Furthermore, our results show the impact of TRPA1 and TRPV1 agonists on cells proliferation and survival.

TRPA1 and TRPA1 have mostly been studied for their effects as nociceptor and chemosensor in sensory neurons. Both channels are expressed in the small Aδ- and unmyelinated sensory C-fibers, where they mediate pain and neurogenic inflammation [240]. Later studies reported the expression of these channels in non-neuronal cells such as in synoviocytes, chondrocytes, lung epithelial cells, and smooth muscle cells [241], [242]. However, their physiological functions are not yet fully understood. In addition, these channels have been shown be highly expressed in different tumors, for example, expression of TRPV1 has been verified in human pancreatic cancer and squamous cell carcinoma of the human tongue, prostate carcinoma, and breast cancer [243]. TRPA1 is highly expressed in tumors such as pancreatic adenocarcinoma, nasopharyngeal carcinoma, and prostate cancer-associated fibroblast cell cultures. Several studies also showed TRPA1 and to a lower level, *Trpv1* mRNA expressions in keratinocytes, and proposed their possible roles in cell growth, survival, inflammation, and abnormal proliferative processes [44]. In line with these data, our study shows the expression of *Trpa1* mRNA in OS K7M2 cells but no expression of *Trpv1* was observed.

The role of TRPV1 and TRPA1 in the tumorigenic process have been linked to its effect on cell cycle progression, which is a hallmark of cancer. Cancer progression is associated with the inhibition of pathways leading to apoptosis which results in uncontrolled cell growth and proliferation. The possible role of these channels in cancer formation and growth is due to the important role the Ca^{2+} signaling plays in these molecular mechanisms such as gene transcription, cell proliferation, differentiation, migration, invasion, and apoptosis [244]. TRP channels are known to be Ca^{2+} permeable ion channels, where they increase the intracellular Ca^{2+} concentration either by inducing Ca^{2+} influx through the plasma membrane or by releasing the stored Ca^{2+} from the mitochondria and the endoplasmic reticulum. This activates Ca^{2+}-dependent signaling pathways including the mitogen-activated protein kinase/extracellular signal regulated kinase (MAPK/ERK) and phosphatidylinositol 3-kinase/Akt Protein Kinase B pathways. Ca^{2+} influx into the tumor cell will activate calmodulin, leading to activation of ERKs, which can further stimulate several regulatory targets in the cytoplasm that have key regulatory roles in cell cycle progression,

survival, and nuclear signaling including MAPK interacting protein kinase, MAPK-activated protein kinases ribosomal S6 kinase, mitogen and stress activated protein kinase, and the protease calpain [244], [245].

Data linking TRPA1 and TRPV1 channels in carcinogenesis is controversial. Activation of TRPA1 in glioma cells led to mitochondrial damage and ultimately to cell apoptosis [219]. Treatment of breast cancer cells with AITC lead to mitochondrial-mediated apoptosis via cytochrome-c, activation of caspase 3 and 9, and the release of apoptosis inducing factor and endonuclease G [246]. Moreover, experimental reports showed that TRPV1 stimulation had tumor suppressor effects in melanoma and colon cancer [247]. Capsaicin induced cell toxicity and death in human OS MG63 cells via TRPV1-dependent and TRPV1-independent signaling pathways [238]. In contrast, others reported that these channels have no effect on cancer, or they may promote cancer growth and metastasis. For example, there was no increase in papilloma growth in two-stage skin cancer mice model with desensitized TRPV1 [244]. The TRPV1 antagonists AMG-980 and SB-705498 did not show any effect on skin carcinogenesis [244], [248]. Furthermore, TRPA1 is shown to play a key role in tumor angiogenesis, thus it activated neovascularization in vivo and increased epithelial cell migration and tubule formation in vitro [249]. Taking these data together that the effect of TRPA1 and TRPV1 activation greatly depends on the cancer cell types and the complex sensory-vascular-immune-tumor interactions in the cancer microenvironment.

In our study, AITC induces Ca^{2+} influx in K7M2 cells and decreases their viability in a dose-dependent manner demonstrating the possible functional expression of TRPA1 where its activation-induced effect is proposed to be through Ca^{2+}-mediated mechanisms. In addition, the TRPA1 antagonist, HC-030031, was able to reverse both the Ca^{2+} influx response and the reduction of cell viability to a small but statistically significant extent. These results are in agreement with previous mentioned reports and with many others showing that a high dose of AITC decreased cell viability, increased DNA damage and inhibited cell migration in HepG2 human hepatocellular carcinoma cells [250] and reduced cell proliferation of breast [246], bladder [251] and cervical cancer cells [252]. Our results show that AITC may inhibit OS cell survival through TRPA1-dependent pathway.

Recent studies have demonstrated that capsaicin induced apoptosis and suppressed tumorigenesis through TRPV1-dependent stimulation of excessive Ca^{2+} influx and TRPV1-

indepenent pathways. In line with these reports, we found that 48 hours capsaicin treatment induced a significant concentration-dependent loss of viability in K7M2 cells. Nevertheless, capsaicin did not have any effect on Ca^{2+} influx in these cell lines and the TRPV1 antagonist, capsazepine was not able to abolish the capsaicin-induced reduction in cell viability. The 48-hour incubation period for both AITC and capsaicin was chosen as there were no cell viability changes upon 24 hours incubation. These data suggest that capsaicin exerts its cytotoxic effect on these cells through TRPV1-independent pathways.

The limitation of the present study is that the expression levels and receptor functionality were only determined in the mouse K7M2 cell line. Since this cell line does not mimic all features of OS, it is difficult to conceive from these results that these channels might be a reliable therapeutic target. However, OS is an extremely malignant cancer with a poor prognosis and resistant to therapies. Therefore, inhibiting tumor cell growth and proliferation by modulating TRPA1 might be a promising molecular tool for further investigations.

In conclusion, our results demonstrate the expression levels of TRPA1 channels in K7M2 cells and suggest that alteration in their activities may reduce OS cell viability, apparently by affecting intracellular Ca^{2+} levels and triggering related signaling pathways. Clinically useful modulators might be promising therapeutic agents. The presumed role of these endogenous channels and activators opens new perspectives for the study of these channels in OS cells and even in non-tumoral bone cells.

III. Testing novel analgesic and anti-inflammatory drug candidates on SST_4 receptor activation and cAMP signaling

1. Background

1.1 Somatostatin receptor 4

Both SST_2 and SST_4 are expressed in pain signaling pathways and are believed to be involved in SST-induced analgesia [253]. However, SST_2 activation is associated with several side effects like inhibiting growth hormone release from rat pituitary cells [254], [255] and insulin secretion [256]. In addition, all SST receptors, except SST_4, are subject to acute desensitization [257]. Also, the SRIF2 receptors are not readily internalized, in contrast to the SRIF1 receptors which undergo rapid and subsequent internalization. For example, exposure of primary neuronal cultures to SST-14 led to progressive loss of SST_2 receptors but not SST_4 receptors [258].

After our team discovery which showed that SST is released from the activated capsaicin-sensitive sensory nerve endings into the systemic circulation leading to anti-inflammatory and anti-hyperalgesic actions in distant body parts, SST receptors emerged as novel analgesic and anti-inflammatory drug targets [10], [259]. Several studies have provided evidence on the significance of SST_4 as a potential target for novel anti-inflammatory and analgesic therapy without endocrine actions. However, to overcome the drawback of the short half-life of SST, great efforts have been and are being put on the development of selective SST_4 agonist with a longer half-life. These effects were reported with synthetic heptapeptide agonist, TT-232, exerting its action on the SST_1 and SST_4 that are located on both immune cells and primary sensory neurons [17], [260]. In vivo intravenous administration of TT-232 in anti-nociceptive effects that were not observed with octreotide [16]. In addition, TT-232 reduced mechanical hyperalgesia in diabetic neuropathy [27] and arthritis models [17]. J-2156, a highly potent, selective, and non-peptide SST_4 superagonist attenuated the sciatic nerve ligation-induced neuropathic mechanical hyperalgesia, adjuvant-evoked chronic inflammatory mechanical allodynia, and the nocifensive behavior in the second phase of the formalin test [261]. In addition, J-2156 reduced the release of pro-inflammatory and pain-related neuropeptide from the peripheral terminals of the peptidergic sensory neurons and suppressed the neurogenic and non-neurogenic inflammatory processes chronic arthritic model [51], [262]. Likewise, SST_4 knockout mice exhibited more severe neuropathic hyperalgesia and inflammatory pain compared to wild types in different pain and inflammatory in vivo models

[263]. Therefore, different pharmaceutical companies considered SST_4 as a target of interest for the development of novel drug candidates to treat inflammation and chronic pain.

The molecular mechanism behind this function is still not fully elucidated. All five subtypes of SST receptor are coupled to adenylyl cyclase inhibition and therefore reduce cellular cAMP levels [264]. Lower cAMP levels may prevent the activation of different protein kinases or alter the function of different ion channels, hence reduce pain. SSTs also activate various types of potassium channels including the G-protein inward rectifying potassium channels (GIRK) [265]. This leads to membrane hyperpolarization and reduction in spontaneous action potential formation which inhibits cell excitability and pain [266]. Besides that, SSTs inhibit depolarization by inhibiting voltage-gated Ca^{2+} channels (VGCC), thus inhibiting cell excitability and release of neurotransmitters and peptides [267]. In addition, TRPV1 and ankyrin 1 TRPA1 channels have an important role in nociception. Activation of Gαi proteins prevents channel sensitization keeping the threshold needed for channel activation high, therefore lead to channel inhibition [268]. All these effects are mediated either through direct binding or by phosphorylation and channel trafficking [269] as presented in Figure 18.

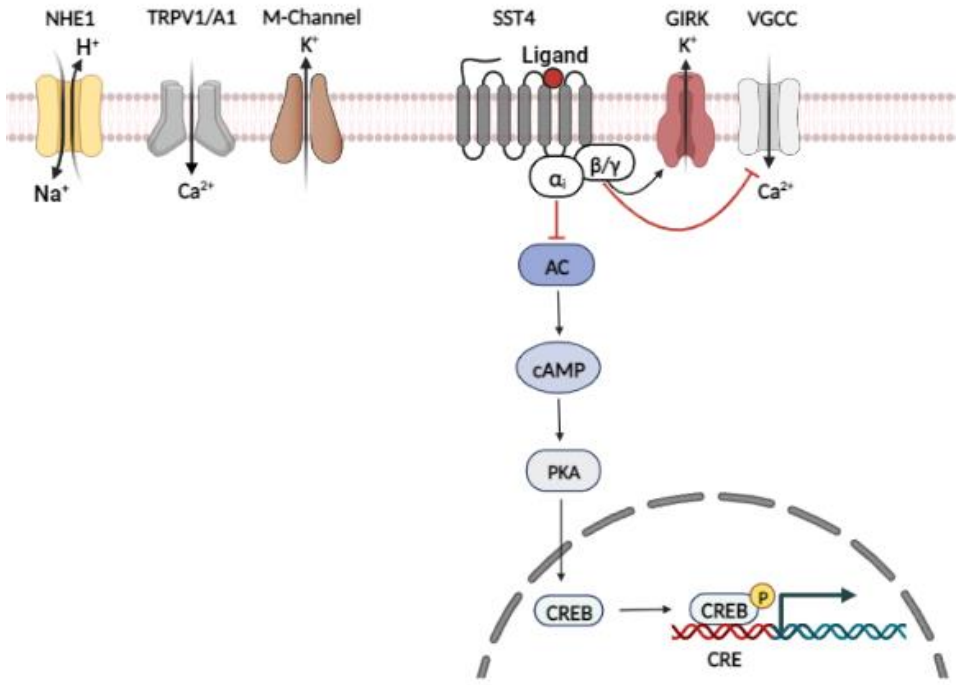

Figure 18. SST_4 signaling leads to inhibition of CREB-regulated gene transcription. This figure was created with Biorender.com.

Based on these data, it is clear that SST_4 is an attractive target for the development of anti-inflammatory and analgesic drugs. Our group has synthesized and patented novel pyrrolo-pyrimidine molecules (Compound 1, Compound 2, Compound 3, Compound 4) (Figure 19), Our previous studies showed that these compounds have maintained interaction with the amino acids of the high affinity binding pocket similar to J-2156 and were effective in G-protein activation assay. Furthermore, a single oral administration of a low dose of these compounds to mice inhibited chronic neuropathic mechanical hyperalgesia [270]– [272]. In this dissertation, we show the binding data of these compounds to SST_4 by means of inhibiting cAMP levels in CHO cells to gain a greater understanding of the molecular mechanisms underlying the role of SST_4 agonists in analgesia.

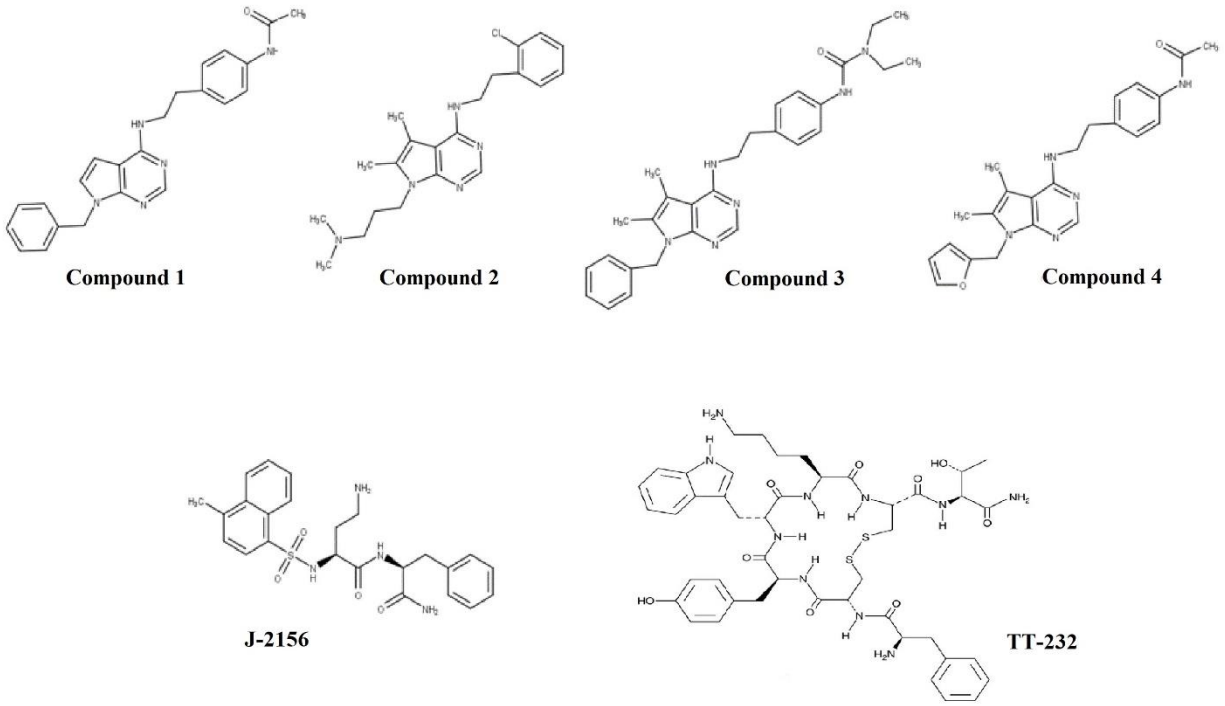

Figure 19. Structures of the tested new pyrrolo-pyrimidine ligands (upper panel), and the high affinity reference molecules (lower panel). Unpublished data.

2. Aims of the study

Since the in vivo analgesic and anti-inflammatory effects of our novel compounds were earlier demonstrated, we aimed to gain a greater understanding of the molecular mechanisms underlying the role of SST_4 agonists in analgesia by:

(I) Investigating if these effects are through direct or indirect activation of SST_4.

(II) Building up and performing a high-throughput cAMP in vitro screening assay, to detect the interaction of these compounds with SST_4.

(III) Confirming the effectiveness and validity of this assay by testing known SST_4 agonists (J-2156, TT-232) on CHO-SST_4.

(IV) Testing our novel pyrrolo-pyrimidine compounds comparing their effect to the reference agonists

(V) Testing the potential selective SST_4-activation by ruling out the effect on CHO-SST_2 cells.

3. Methods

3.1 Cell culture

Human SST_4 and SST_2 receptor- expressing CHO-K1 cells (DiscoverX, Fremont, CA, USA) were cultured in 75T flask in DMEM/Nutrient Mixture F-12 Ham (DMEM/F12, Thermo Fisher Scientific, USA) supplemented with L-glutamine, 10% FBS, 1x penicillin/streptomycin, and 800 µg/ml selection antibiotic G418. They were kept at 37 °C, 5% CO_2 incubator until 70-80% confluency.

3.2 cAMP accumulation assay

The accumulation levels of cAMP were measured using the DiscoverX HitHunter™ cAMP assay kit (DiscoverX, Fremont, CA). Initially, 10 mM stock solutions of all compounds were prepared in DMSO (Sigma) and kept at -20 °C for future use. Cells were seeded into white 96-well assay plates at a density of 20,000 cells/well with 100 µl cell plating reagent/ well (DiscoverX, Fremont, CA) and incubated overnight at 37 °C, 5% CO_2. In the following day, cell plating reagent (DiscoverX, Fremont, CA) was changed to PBS. A series of serial dilutions (10 µM – 10 pM) of compounds with PBS containing the phosphodiesterase inhibitor, rolipram, and the adenylyl cyclase stimulator, forskolin (10 µM) was performed. Cells were then treated with different concentrations of SST_4 agonists for 30 minutes at 37°C. All measurements were taken in duplicates. Once agonists treatment was completed, several incubations with the assay reagents (DiscoverX, Fremont, CA) were done at RT. The chemiluminescent signal proportional to the cAMP concentration was observed using a PerkinElmer EnSpire Alpha plate reader. The data was expressed as percentage of cAMP levels proportional to the forskolin response (Figure 20).

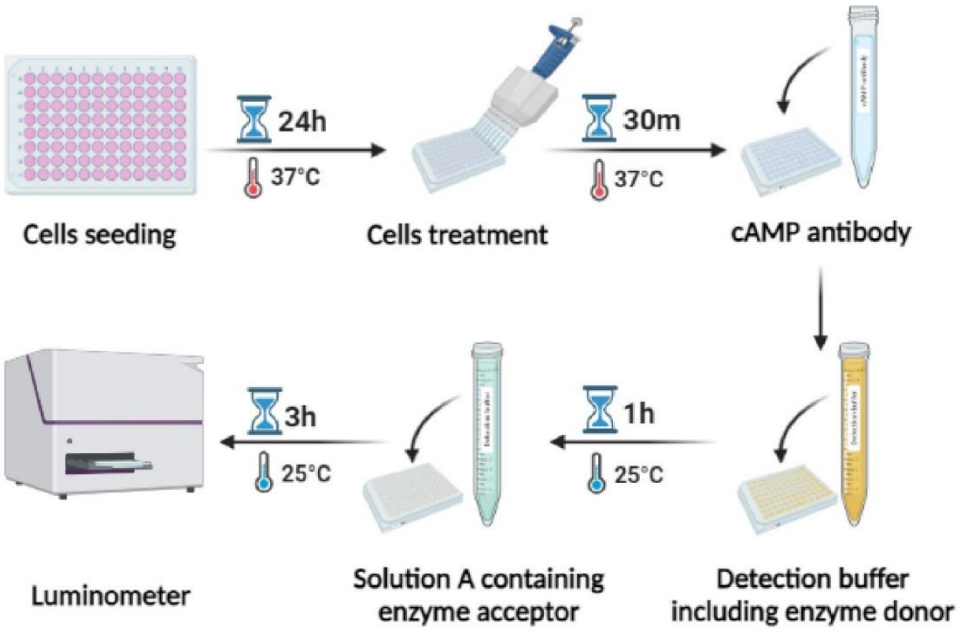

Figure 20. Schematic presentation of cAMP assay protocol. This figure was created with Biorender.com.

3.3 Statistical analysis

Calculations and curves were made using GraphPad Prism (GraphPad Prism version 8.0.1). Nonlinear regression fit using the sigmoidal dose–response equation was performed. All results are expressed as means ± S.E.M. Values of EC_{50} and E_{max} were compared using unpaired t-test with Welch's correction. The level of statistically significant differences was set as $P < 0.05$.

4. Results

J-2156 and TT-232 showed a clear dose-response inhibition of the forskolin-stimulated cAMP production in SST_4-expressing CHO cells (Figure 21. A, B) with EC_{50} of 681.4±63.91 pM and 371.6±58.03 nM, respectively, and E_{max} of 90.53±1.776% and 78.63±2.636%, respectively at a concentration range of 10 pM - 10 µM. However, testing the dose-response cAMP inhibition produced by the pyrrolo-pyrimidine molecules showed that under our conditions, these novel ligands could not inhibit forskolin-stimulated cAMP on SST_4-expressing CHO cells (Figure 21. C, D, E, F). In addition, cAMP inhibition was not observed with any reference or tested

compounds on SST_2-expressing CHO cells (Figure 22) proving the selectivity of the SST_4 reference compounds.

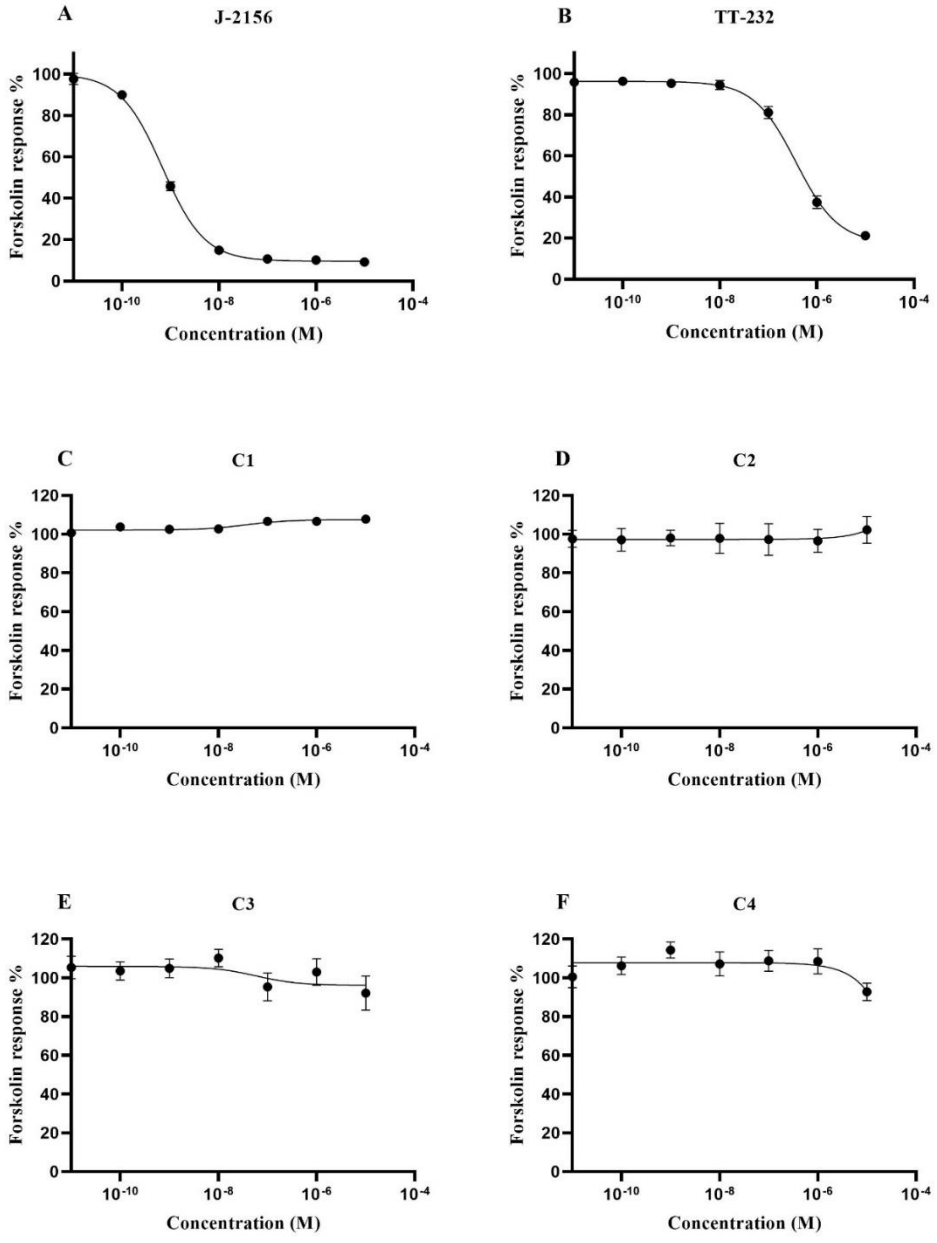

Figure 21. Concentration-response curves of the tested compounds on SST_4-linked cAMP accumulation. J-2156 and TT-232 show SST_4-induced inhibition of cAMP accumulation. The cAMP assay is carried out on CHO-K1-SST_4 cells**.** Each data point is means ± SEM expressed as a percentage of the 10 μM forskolin response.

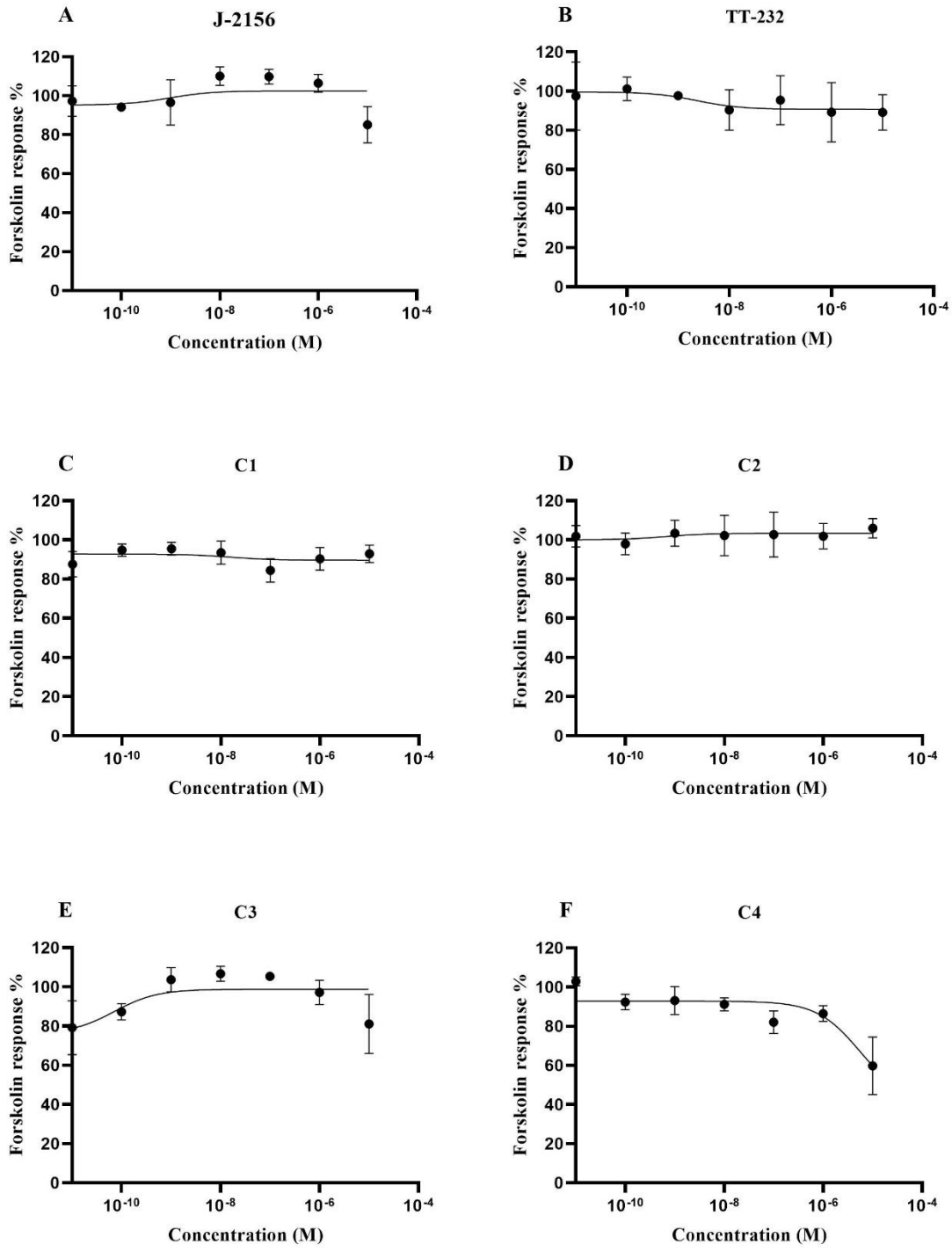

Figure 22. Concentration-response curves of the tested compounds on SST_2-related cAMP accumulation. No SST_2-mediated inhibition of cAMP accumulation is observed by any of tested compounds. The cAMP assay was carried out on CHO-K1-SST_2 cells**.** Each data point is means ± SEM expressed as a percentage of the 10 μM forskolin response.

5. Discussion

This study is built on the knowledge that SST has anti-inflammatory and analgesic characteristics that are believed to be predominantly mediated by SST_4 receptor activation [16]. This was demonstrated by implementing *in vivo* pain model confirming the anti-inflammatory and anti-nociceptive effects of TT-232 without showing any of the endocrine effects [273]. These studies were later further confirmed with J-2156 as it has higher SST_4 affinity and selectivity [51], [261], [274], [275]

Our reference selective agonists, J-2156 and TT-232, were able to strongly inhibit forskolin-stimulated cAMP levels in a dose-dependent manner in SST_4, but not in SST_2-expressing CHO cells. This suggests the G_i protein activation, which is known to be coupled with SST_4 receptors. Several studies proposed that inhibition of cAMP pathway reduces pain-like behaviors in animal models [276], and that targeting adenylyl cyclase or protein kinase A may relieve pain with minimal side effects [277]. Previously, our novel pyrrolo-pyrimidine compounds showed SST_4 interaction *in silico*, SST_4-associated gamma-GTP binding *in vitro* by the G-protein activation assay, and analgesic and anti-inflammatory effects *in vivo* rat and mouse models [270]–[272]. However, they failed to produce any detectable SST_4-associated cAMP inhibition. This contradiction can be explained by the possible other SST_4-linked signaling mechanisms independent from cAMP inhibition. Previous studies showed that SST_4 mediated inhibition of GIRK, VGCC, in rat dorsal root ganglion (DRG) sensory neurons [275], and inhibited VGCC in rat retinal ganglion cells [278]. In addition, Ca^{2+} imaging experiments demonstrated that J-2156 was able to inhibit voltage induced Ca^{2+} influx on DRG culture [274].

It was thought before that SST mechanism of action may be through an agonistic effect at the mu opioid receptors. However, naloxone could not antagonize the SST-induced inhibition of nociceptive neurons [279]. This suggested that SST induces analgesic effects independent of the opioidergic neurotransmitter system. Ion channels, including GIRK, VGCC, and TRP channels play an important role in the transduction and transmission of pain signals and could contribute to SST_4-induced analgesic effects. [11], [266], [269], [280]. The activity of these channels can be mediated by direct interactions or through GPCRs as shown before with several analgesics [212], [281]– [283]. Although the mechanism for SST_4 anti-nociceptive and anti-inflammatory action is still not fully understood, SST's role in other physiological and pathological conditions could guide us to the possible processing pathways via ion channels. SST inhibited glucose-induced

electrical activity in pancreatic β-cells via combined activation of GIRK channels and ATP sensitive potassium channels, an effect that was mediated by the SST_5 receptor [284]. In addition, SST resulted in hyperpolarization of neuronal endocrine cells through GIRK channel activation [285]. Indeed, strong activation of inward potassium currents by SST-14 was observed in cells co-expressing SST_1-SST_5 receptors with a subunit of the GIRK1 ion channel in xenopus oocytes [286]. Whole-cell voltage clamp recording using the SST_4 receptor selective agonist J-2156 [274] and the potent GIRK blocker tertiapin Q [287] confirmed functional links of the SST_4 receptor to GIRK channels. These studies were further confirmed when conducted on cultured DRG neurons in order to make this relevant to the analgesic effects. J-2156 evoked a dose dependent increase in potassium current in DRG neurons, which was significantly reduced by tertiapin Q [275]. This provided evidence that J-2156-induced current was because of GIRK activation.

VGCCs are also involved in several disease conditions and play a vital role in the DRG neurons. Inhibition of Ca^{2+} channels lead to slower cell depolarization and therefore modulate DRG neurons excitability and reduce nerve transmission. Besides that, low intracellular Ca^{2+} level would also affect secondary messenger actions which alters neuropeptide release [269]. J-2156 which could inhibit the VGCCs also inhibited substance P and CGRP release from trachea cells [51]. Moreover, Ca^{2+} imaging experiments demonstrated that J-2156 could inhibit depolarization-induced Ca^{2+} influx in DRG neurons [274]. Similar effect was shown with another selective SST_4 receptor agonist (L-803,087) on retinal ganglion neurons [278]. This suggests that VGCCs inhibition is a possible mechanism behind this effect and can be responsible for SST_4 receptor agonist's analgesic effects.

TRP channel family is known to be associated with pain onset and could be affected by activation of SST_4 receptor [288], [289]. TRPV1 channels are mainly expressed in nociceptive sensory neurons, where they play key roles in the transmission of painful stimuli. Application of SST on skin nerve preparation of peripheral nociceptors, strongly inhibited capsaicin induced TRPV1 activity and prevented desensitization [289]. The selective SST_4 agonist, TT-232, was able to significantly reduce nociceptive and inflammatory responses induced by capsaicin *in vivo* [260], showing that SST_4 receptor agonists modulate TRPV1 activity. Inhibition of TRPA1 channel may also be affected by SST_4 receptor, J-2156 compound inhibited 1% mustard oil induced Evans blue leakage in paw skin of rats and ear oedema in mice [51].

Another mode of action might be through dimers formation, where different subtypes of receptors bind together as heterodimers leading to modifications in the signaling and internalization of these receptors [290]. This was demonstrated with SST_4 and δ-opioid receptors heterodimer which led to synergistic effects of multiple downstream signaling pathways [291]. But the significant role for SST_4 receptor selective agonists in reducing pain and the inability of opioid antagonists to reverse the effect, propose that dimers formation is not vital for the SST_4 associated analgesic effects [292].

Any of these mechanisms may contribute to the analgesic action of our pyrrolo-pyrimidine compounds, which need to be further investigated and established.

We could successfully build and validate the SST_4 receptor activation by cAMP *in vitro* assay, which can be further used for screening of novel agonists as analgesic and anti-inflammatory candidates avoiding the endocrine side effects of SST mediated by the SRIF1 receptor group. Based on the effect of the reference compounds J-2156 and TT-232, we standardized a relatively high-throughput screening assay on 96-well plate for ligand testing.

Our novel pyrrolo-pyrimidine compounds have the ability to control neuropathic and inflammatory pain, but the cellular mechanism of action needs further invistigations and elucidations.

Summary of the novel results and conclusions

In the experimental context, cell culture is mostly needed to create a model system through which scientists can study the basic cell biology, disease mechanisms, or investigate the target and toxicity of novel drug candidates. Advantages of using cell culture includes the homogeneity of specific cell type or clonal cell populations, the ability to control genes and molecular pathways, the possibility to avoid interaction of genetic or environmental variables in a well-defined culture, and the generation of a highly reproducible and consistent data [293]. Cell lines have improved the scientific research as they are currently used in different studies for drug testing, synthesis of biological compounds, vaccine production, toxicity studies, antibody production, gene function studies. However, it should be stated that *in vitro* cell cultures, both primary cells and cell lines have the disadvantage of being studied in the absence of their surrounding environment that usually includes the interference of different parameters and interactions with different cell types that may affect the hypothesis being tested in a specific study [294], [295].

This thesis show three studies testing various candidates/targets for inflammation, pain, and cancer emlpoying different cell-based assays summarized as follows:

- We first investigated the effects of ATRA and its novel diphenylacetylene-based derivatives on cell viability and genotoxicity in the widely used, rapidly proliferating CHO cell line. DC360 is a fluorescent analogue, and DC324 a non-active analogue of DC360. EC23, DC712, DC645, DC540, and DC525 are more stable, promising ATRA analogues. There was no cytotoxic effect. However, all compounds provoked DNA migration similar to ATRA or to a greater extent in the case of DC324, DC360 and EC23 particularly at higher concentrations. DC360 was the most toxic agent, which can be due to the enamine function of the hydrophobic region (high Log P structure). Our results demonstrate RAR or RXR-independent genotoxicity, thus testing the activation of TRPV1 and TRPA1 channels as a potential molecular mechanism for this effect and for retinoids-related irritations and painful side effects is considered in our future research.

- We also investigated the functional expression of TRPA1 in OS tissues and cell lines. TRPA1 is expressed in human and mouse tissues as well as in OS K7M2 cells and the TRPA1 agonist AITC induces radioactive calcium uptake and reduces cell viability. This

effect was reversed by the TRPA1 antagonist HC-030031. Usually, TRPA1 and TRPV1 are co-expressed in both neuronal and non-neuronal cells where they may affect each other's function. Therefore, we investigated the TRPV1 expression in these OS models. TRPV1 shows expression in human and mouse tissues but very low expression in K7M2 cells, this may be due to the differences in tumor microenvironment between tissues and cell lines. Capsaicin does not cause significant Ca^{2+} uptake but reduces K7M2 cell viability without antagonistic effect of capsazepine. This study suggests a decrease in OS cell growth and survival by TRPA1-dependent and TRPV1–independent pathways. Different apoptosis assays are planned to be conducted to reveal the possible molecular mechanisms behind the effects of AITC and capsaicin.

- We validated the SST_4 receptor as a promising target for the development of anti-inflammatory and analgesic drugs by showing the binding of both J-2156 and TT-232 by means of cAMP reductions. However, our results show that our pyrrolo-pyrimidine compounds do not affect cAMP levels and suggest that they may exert their anti-inflammatory and analgesic effects through different signaling pathways. The molecular mechanism of these compounds still needs to be investigated further while other novel molecules are being synthesized and assessed in our laboratory.

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List of publications

First author publications connected to research topic:

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Participations in other conferences

Second Symposium on Super-resolution and Advanced Fluorescence Microscopy and István Ábrahám Memorial Workshop, 2022, Pécs.

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Article

Synthetic Diphenylacetylene-Based Retinoids Induce DNA Damage in Chinese Hamster Ovary Cells without Altering Viability

Lina Hudhud [1], David R. Chisholm [2], Andrew Whiting [2], Anita Steib [1], Krisztina Pohóczky [1,3], Angéla Kecskés [1], Éva Szőke [1,†] and Zsuzsanna Helyes [1,*,†]

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Abstract: All-trans-retinoic acid (ATRA), the active metabolite of vitamin A, plays a pivotal role in cell differentiation, proliferation and embryonic development. It is an effective therapy for dermatological disorders and malignancies. ATRA is prone to isomerization and oxidation, which can affect its activity and selectivity. Novel diphenylacetylene-based ATRA analogues with increased stability can help to overcome these problems and may offer significant potential as therapeutics for a variety of cancers and neurodegenerative diseases, including amyotrophic lateral sclerosis. Here, we investigated the effects of these retinoids on cell viability and genotoxicity in the widely used model system of the rapidly proliferating Chinese hamster ovary cell line. DC360 is a fluorescent ATRA analogue and DC324 is a non-active derivative of DC360. EC23, DC525, DC540, DC645, and DC712 are promising analogues with increased bioactivity. The cytotoxic activity of the compounds was evaluated by ATP assay and DNA damage was tested by comet assay. No cytotoxicity was observed in the 10^{-6}–10^{-5} M concentration range. All compounds induced DNA migration similar to ATRA, but DC324, DC360 and EC23 did so to a greater extent, particularly at higher concentrations. We believe that retinoid receptor-independent genotoxicity is a general characteristic of these compounds; however, further studies are needed to identify the molecular mechanisms and understand their complex biological functions.

Keywords: retinoids; all-trans-retinoic acid; genotoxicity; DNA damage; ATP assay; comet assay

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1. Introduction

Retinoids are vitamin A derivatives that play an important role in cell differentiation, proliferation, and apoptosis [1]. They are approved as chemopreventive and chemotherapeutic agents [2]. However, their use has been limited due to a lack of understanding of their complex signaling pathways and potential systemic toxicity [3,4].

All-trans-retinoic acid (ATRA) is the major vitamin A metabolite [5] and has essential roles in different biological processes during both embryogenesis and adult life [6]. Therefore, it is indicated for the treatment of various skin conditions and cancers, either alone or in combination with other cytostatic drugs. ATRA is prone to isomerization and oxidation, which can lead to significant changes to the compound's activity and selectivity [6,7]. These changes could be of special interest when studying its toxic effects on non-tumor cells. Synthetic analogues with increased chemical stability could help to overcome these problems and may offer significant potential as pharmaceutical agents with lower toxicity.

The broad side effect profile of retinoids, including mutagenesis, has long held back their clinical use, particularly their systemic administration. The genotoxicity of retinoids is controversial and seems to be dependent on cell types, treatment concentrations and duration, as well as experimental conditions. ATRA did not induce structural aberrations within the chromosomes of human embryonic palatal mesenchymal cells [8]. In contrast, certain retinoids, including 13-cis-retinoic acid, caused sister chromatid exchanges in human diploid fibroblasts [9,10]. In addition, retinol induced chromosomal aberrations in human lymphocytes [11], as well as enhanced DNA single strand breaks and fragmentation, and the formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine, the major DNA oxidation product associated with oxidative stress, in cultured Sertoli cells [12]. Results of a recent clinical trial showed that supplementation with β-carotene (pro-vitamin A) increased the incidence of lung cancer in smokers [13], whereas it had earlier been demonstrated that people with higher serum β-carotene levels had a lower risk of cancer [14]. In addition, ATRA and its steroid analog EA-4 induced micro-nucleation by chromosome breakage in the C2C12 mouse myoblast cell line and HL-60 human acute myeloid leukemia cells [15].

We synthesized novel diphenylacetylene-based ATRA derivatives with a wide range of structural variations (Figure 1) in order to increase their stability and reduce vulnerability to oxidation and isomerization, which might affect their activity and safety. DC360 exhibits fluorescence when activated by visible light (380-420 nm); DC324 is a DC360 derivative with an extended structure that prevents binding to retinoid-related nuclear receptors and transport proteins; hence, it is a non-active fluorescent retinoid [3]. EC23, DC525, DC540, DC645 and DC712 are synthetic ATRA derivatives with increased receptor binding and bioactivity [3,16,17]. These synthetic ATRA analogues were proven to cause differentiation in, for example, neuroblastoma cell lines, similar or more strongly than ATRA [18,19]. They have significant potential as therapeutics for a variety of cancers and neurodegenerative diseases including amyotrophic lateral sclerosis (ALS) [20].

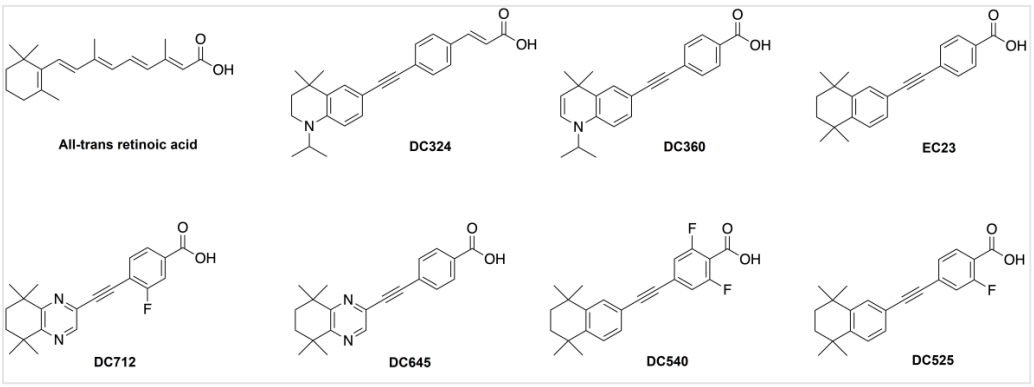


Figure 1. Chemical structures of ATRA and its novel synthetic analogues.

Since there are no data about the potential cytotoxicity and genotoxicity of these novel compounds, here we investigated their effects on cell viability and DNA stability. The aim of this study was to examine the effect of these compounds on normal cells as they have the potential for systemic treatment of various, non-cancerous diseases. Chinese hamster ovary (CHO) cells were chosen, as they represent a widely used model system for the comet assay [21–23], which are easy to handle and highly tolerant to variations in pH, oxygen levels and temperature.

2. Results

2.1. ATRA and Its Synthetic Derivatives Do Not Exert Cytotoxic Effects on CHO Cells

Neither ATRA nor any of the synthetic retinoic acid derivatives induced cytotoxicity at a concentration of 10^{-6} M (Figure 2A). DC324 exerted a small, but statistically significant decrease in cell viability compared to the vehicle control in 10^{-5} M, but it was still greater than the 70% relative survival that is required for the comet assay (Figure 2B).

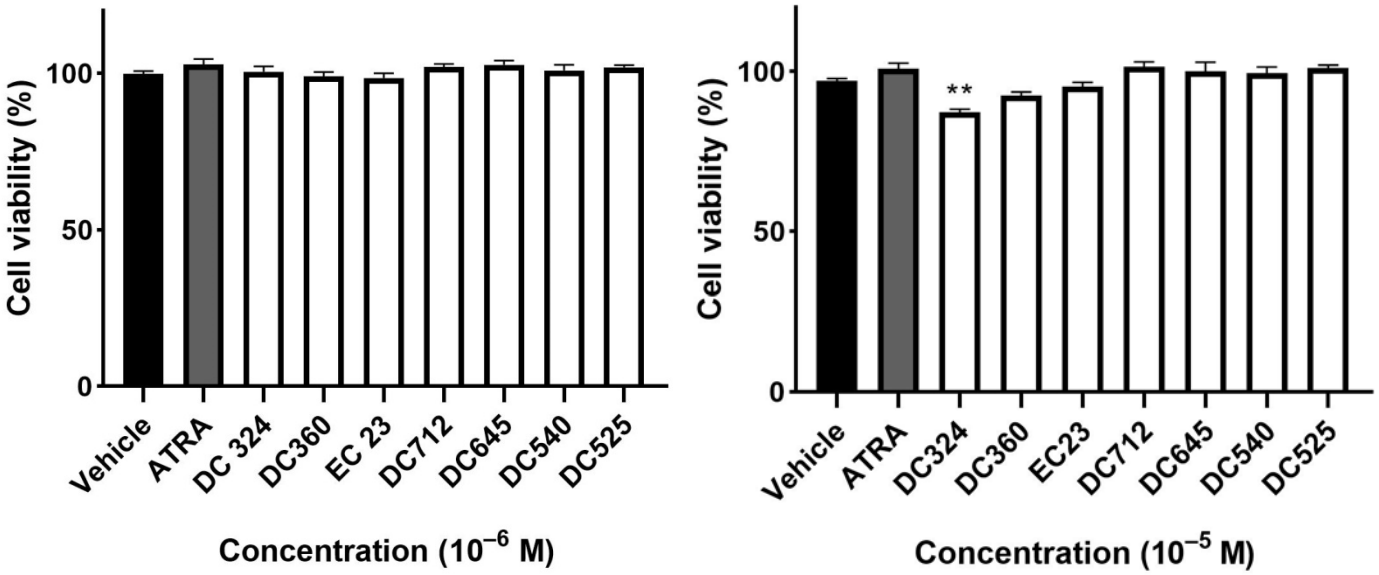


Figure 2. Viability of CHO cells. Cells were exposed to ATRA or its synthetic analogues in 10^{-6} M (**A**) or 10^{-5} M (**B**) for 24 h and cell viability was detected by the ATP viability assay as a percentage of the vehicle-treated control. Each column represents the mean ± SEM of n=12 experiments performed in two independent series (** $p < 0.01$ vs. vehicle).

2.2. Synthetic Derivatives Induce Similar or Greater DNA Damage as Compared to ATRA

Representative fluorescence images of CHO cell comets treated with ATRA, DC324, DC360, EC23, DC712, DC645, DC540 and DC525 compared to the vehicle control and H_2O_2 in 10^{-5} M concentration are shown in Figure 3.

H_2O_2 (10^{-5} M) as a positive control significantly increased, by almost double he %tail DNA compared to the vehicle control. ATRA also significantly increased %tail DNA at 10^{-5} and 10^{-6} M but not at lower concentrations; the damaging effect was much less than H_2O_2. All tested concentrations of DC360 and the 10^{-6} M concentration of DC324 significantly increased %tail DNA compared to both vehicle control and ATRA at a similar extent to H_2O_2 (Figure 4A–D). We observed significant elevation of %tail DNA upon EC23 treatment at a concentration of 10^{-7}–10^{-5} M compared to both control and ATRA (Figure 4A–C), but the 10^{-8} M concentration did not induce any alterations (Figure 4D). The highest concentration (10^{-5} M) of DC645 and DC525 significantly elevated %tail DNA compared to both vehicle control and ATRA, while DC712 and DC540 induced a significant increase only compared to vehicle (Figure 4A). At 10^{-6} M, these four compounds showed a significant increase compared to the vehicle control (Figure 4B).

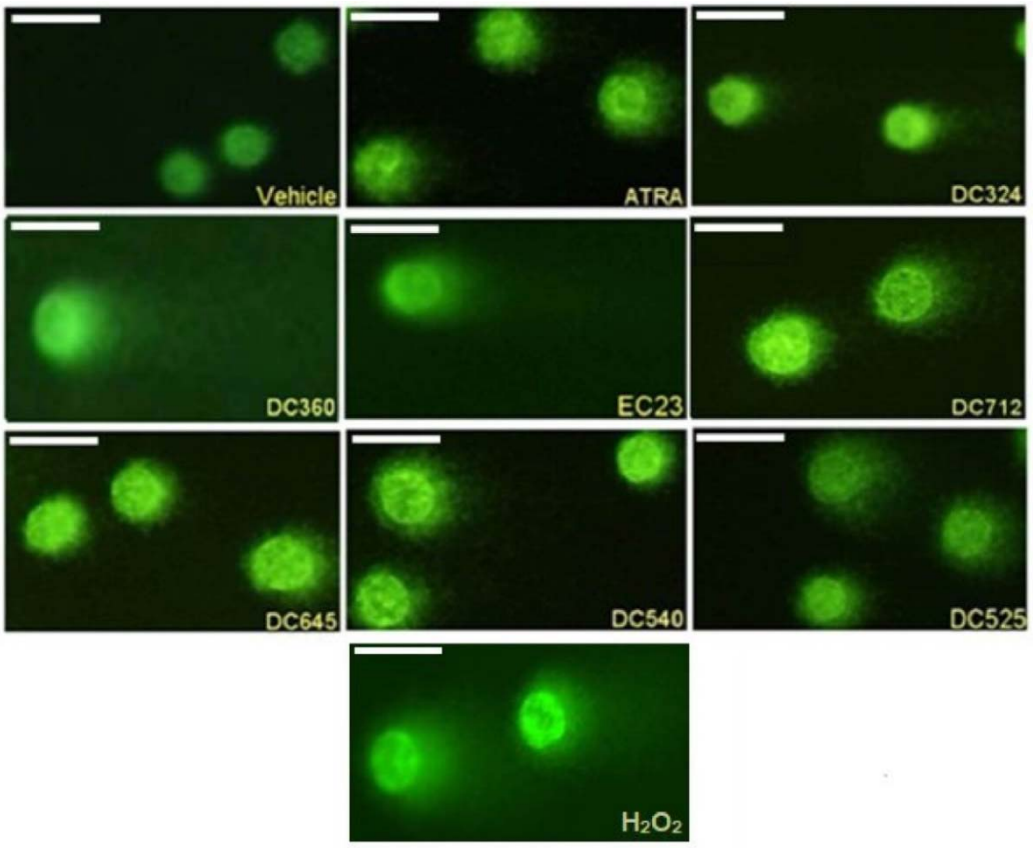
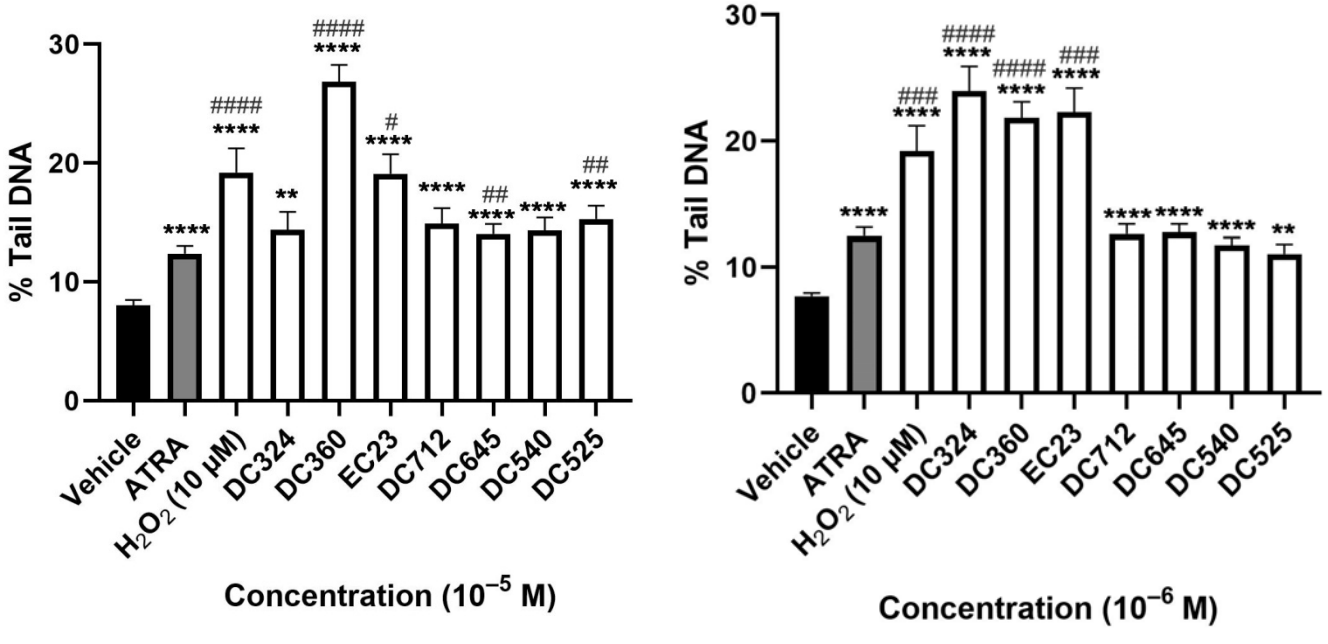


Figure 3. Representative comet images. CHO cells treated for 24 h with vehicle, 10^{-5} M ATRA, DC324, DC360, EC23, DC712, DC645, DC540, DC525, and H_2O_2 for 15 min. Magnification 200×.



(**A**) (**B**)

Figure 4. *Cont.*

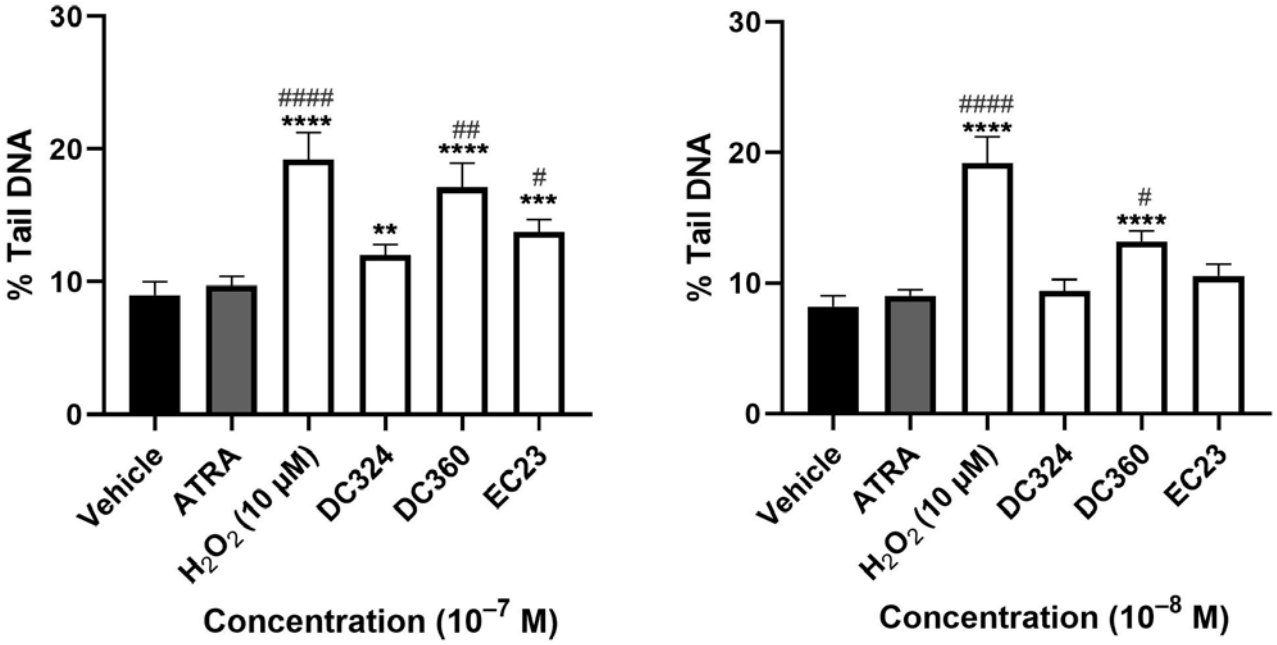


Figure 4. Degree of DNA-damage following retinoid treatments. Columns represent the %tail DNA of CHO cells treated for 24 h with vehicle, 10^{-5} M H_2O_2 as the positive control, and the retinoid compounds (10^{-5} M (**A**), 10^{-6} M (**B**), 10^{-7} M (**C**), 10^{-8} M (**D**)). Each column (Except H_2O_2) demonstrates the mean ± SEM of n= 100 cells derived from two independent series (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs. vehicle), (# $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$ vs. ATRA).

3. Discussion

We present here the first functional results for the effects of seven novel diphenylacetylene-based synthetic retinoids on cell viability and DNA stability. It is demonstrated here that these synthetic ATRA analogues do not induce cytotoxic effects, but cause genotoxicity detected by DNA strand breaks in CHO cells. DC360 showed the greatest increase of %tail DNA even at the lowest tested concentration, and the DNA damaging effects of the other derivatives were mostly similar to that of ATRA.

Different in vivo and in vitro studies have shown that retinoids are effective in prevention and treatment of cancer [24,25], but their toxicity has limited their clinical use [26,27]. A strong relationship between DNA damage and cancer has been investigated, where the induction of chromosome breakage is regarded to be genotoxic and potentially carcinogenic [28]. DNA is very sensitive to a wide range of endogenous and exogenous modifications damaging factors leading to mutations [29]. Genomic DNA damage, such as base modifications, strand cross links and breaks occurs spontaneously in response to chemical or physical mutagens [30,31] and influences transcription, replication and chromosome segregation [32]. When there is DNA damage, specific proteins are activated and the cell cycle is arrested until the damage is repaired [33]. If the damage is irreparable, the cells undergo permanent cell-cycle arrest, senescence or cell death (apoptosis/necrosis) [34]. However, when DNA damage is successfully repaired, apoptosis signaling proteins are not activated and the viability of cells is preserved [35]. Therefore, we believe that our compounds cause repairable DNA damage.

The non-cytotoxic concentration was chosen to test the direct effect of the compounds on DNA damage. ATRA and the synthetic analogues did not, at 10^{-6}–10^{-5} M concentrations, significantly decrease cell viability, except for DC324, but the inhibition was only 13 ± 1%. This is less than the generally accepted guidelines for the comet assay, which recommend testing above 70% viability [36–38]. Therefore, all compounds could be further investigated in the genotoxicity assay.

Our findings regarding the DNA damaging effects of ATRA on CHO cells support previous results in other cell types. For example, ATRA triggered apoptosis in two human hepatoma cell lines, HepG2 and Hep3B, as assessed by flow cytometry [39] and in HL-60 human acute myeloid leukemia cells by typical DNA fragmentation [40]. Moreover, ATRA and its steroid analogue EA-4 caused DNA fragmentation in both C2C12 mouse myocyte cells and HL-60 [41]. ATRA also increased the level of intracellular reactive oxygen species and DNA damage in ARPE-19 cells after exposure to tert-butyl hydroperoxide [42]. All synthetic ATRA analogues induced similar genotoxic effects to ATRA, but DC324, DC360 and EC23 are even more toxic, particularly at higher concentrations. Since the inactive retinoid, DC324, which lacks the ability of retinoid receptor binding, also induced remarkable DNA damage, the observed effects are likely to be independent of the receptor-mediated mechanism. These compounds have the most hydrophobic (high log P) structures and, therefore, they may exhibit stronger off-target interactions with other proteins and DNA species. The enamine function of the dihydroquinoline hydrophobic region of DC360 could also be reactive towards cellular components under certain circumstances; this structural motif may, therefore, be the cause of the stronger genotoxic effect of this compound compared to the others. Further studies are required to elucidate the molecular basis of these genotoxic effects.

Retinol and β-carotene have been proven to have pro-oxidant effects, which might lead to oxidative damage and carcinogenesis [43–45]. They can generate free radicals when supplemented at doses higher than the normal dietary intake, which might lead to an increased incidence of different types of cancer, such as esophagus, oral cavity, pharynx, larynx, stomach, colon and rectum [46]. Stimulation of antioxidant enzyme activities and oxidative damage in primary cultured Sertoli cells was observed upon retinol treatment, which may be due to increased iron uptake and storage and the generation of highly reactive hydroxyl radicals by the Fenton reaction [12,47]. This can cause DNA breaks by directly attacking the deoxyribose [48]. Retinol also changes the organization and function of chromatin, thus altering the on- and off-switching of transcriptionally-active regions of DNA in rat Sertoli cells [49], as well as increasing DNA fragmentation in Chinese hamster lung fibroblasts and inducing DNA breaks, cell cycle progression and increasing the numbers of proliferative foci in terminally differentiated rat Sertoli cells [50]. All of these mechanisms might be involved in the observed genotoxic actions of the novel compounds in CHO cells.

The ATP viability assay is a homogenous, widely accepted method as a valid marker of viable cells. It can detect low numbers of cells (10 cells per well) very rapidly, within 10 min to several hours after adding the reagent [51]. Using this assay, we determined the cytotoxicity of our compounds. When the aim is to clarify the mechanism of cytotoxicity, various apoptosis assays can be performed, such as annexin v and caspase assays [52]. In parallel, the comet assay is an efficient and standard method to quantify the extent of DNA damage at the cellular level and is commonly used for genotoxicity and biomonitoring [53]. However, it has some limitations; for example, it only represents the ratio of the fragmented DNA. Therefore, in order to more precisely understand the molecular mechanisms, other assays such as γH2A.X staining and immunoblotting are needed [54]. Here, we screened the effects of our novel compounds on cell viability and DNA fragmentation without the scope of identifying the mechanisms.

In summary, the novel synthetic diphenylacetylene-based ATRA derivatives are not cytotoxic, but they do induce DNA migration due to DNA strand breaks potentially leading to genotoxicity and genome instability. The fluorescent compound, DC360, shows the most pronounced DNA damaging action. Further studies are needed to identify the molecular mechanisms and understand the complex biological activities of these compounds. Based on the present results, we can clearly state that the retinoid receptor-independent genotoxicity is their general characteristic, which should be considered in later development and applications.

4. Materials and Methods

4.1. Test Compounds

ATRA was purchased from Merck KGaA (Darmstadt, Germany). EC23 and DC324, DC360, DC525, DC540, DC645 and DC712 were prepared according to the published procedures (Figure 4) [3,55,56].

4.2. Cell Culture

The Chinese hamster ovary (CHO-K1) cell line (ATCC, Virginia, USA) was chosen as fast-dividing cells (subculturing rate is 1:4–1:8 according to ATCC product sheet). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 4 mmol L-glutamine, 10% fetal bovine serum (FBS), 1x penicillin/streptomycin (Thermo Fisher Scientific). Cells were kept at 37 °C in a 5% CO_2 incubator.

4.3. Cell Viability ATP Assay

500 µM stock solutions of compounds in absolute ethanol (vehicle) were initially prepared. CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Mannheim, Germany) was used to determine the number of viable CHO cells in culture. CHO cells were seeded in a 96-well plate at a density of 5000 cells/well in Dulbecco's Modified Eagle's Media supplemented with 10% FBS, 4 mmol *L*-glutamine and kept in incubator at 37 °C and 5% CO_2. After 24 h, cells were treated with 10^{-6} M and 10^{-5} M retinoic acid derivatives (prepared in 100 µL of media) and incubated for 24 h. After incubation, 100 µL CellTiter-Glo reagent was added to each well. The plate was placed for 2 min onto an orbital shaker. After 10 min incubation at room temperature, cell viability was measured with a luminescence microplate reader (EnSpire AlphaLISA, PerkinElmer, Inc, Waltham, MA, USA) at an integration time of 0.25–1 s per well. Vehicle treated wells served as negative control. The viability of treated cells was determined by comparing ATP levels of treated wells to vehicle-treated controls. The experiment was repeated twice with 6 wells/compound each time.

4.4. DNA Damage Comet Assay (Single Cell Gel Electrophoresis)

The comet assay is a fundamental method to detect DNA damage. Cells embedded on a slide are lysed to form a nucleoid consisting of a DNA dense core surrounded by a lighter halo [57]. The presence of strand breaks relaxes the DNA supercoiling forming a loop, which is released into the halo during alkaline electrophoresis [58] resulting in comet-like structures [59]. The number of relaxed loops in the tail indicates the number of DNA breaks [38].

Initially, the CHO cells were cultured in 6-well plate Frosted microscopic slides were coated with 0.8% normal melting point agarose (SeaKem®, Lonza) and stored for future use. Cells were treated for 24 h with ATRA, DC324, DC360, EC23, DC712, DC645, DC540, DC525 or hydrogen peroxide (H_2O_2) for 15 min as positive control; vehicle-treated wells were used as negative controls. The tested concentrations of compounds were chosen as non-cytotoxic. In brief, cells were trypsinized, centrifuged, resuspended and mixed well with 0.7% low melting point agarose at 40 °C. A 200 µL sample was transferred onto the pre-coated slide (3 slides/treatment) and covered with a coverslip. Slides were placed at 4 °C for 5 min to allow agarose solidification. Then, coverslips were removed, and slides were incubated in a lysis solution (pH 10) at 4 °C overnight. Electrophoresis was performed at 25 V, 300 mA for 30 min in a cold electrophoresis buffer (pH 13). Slides were then washed with neutralizing buffer (pH 7.5), distilled water, and absolute ethanol (5 min in each solution). Finally, slides were allowed to dry, and then stained with Eco-safe (PacificImage Electronics) stain in the dark at RT. After the removal of excess stain, the slides were covered with 2–3 drops of fluoromount solution (Fluoromount-G™, Invitrogen) and a coverslip. Image acquisition was performed using Olympus BX50 fluorescence microscope with 200× magnification and evaluated with an image analysis software program (OpenComet plugin in ImageJ

software). Two independent experiments were conducted for each treatment in which three slides were used to score different cells. At least 50 cells/ experiment (except for H_2O_2) were selected randomly for the analysis of comet by quantifying the DNA damage as total percentage of DNA in the tail (%tail DNA).

4.5. Statistical Analysis

Statistical analysis was performed by Graphpad Prism (Version 8.0.1). The normality was tested by Shapiro–Wilk test, and, since data were not normally distributed, they were analyzed using the Kruskal–Wallis followed by Dunn's multiple comparisons test. The results are presented as mean ± SEM at $\alpha = 0.05$ level of significance.

Author Contributions: Z.H., A.W. and É.S. designed the study. L.H., A.S., K.P., A.K. and D.R.C. performed parts of experiments, interpreted the data, and performed data analysis. L.H., Z.H., A.W., A.K. and É.S. drafted the manuscript and revised it critically for intellectual content. All authors read and approved the final version of the manuscript before submission.

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Conflicts of Interest: The authors declare no conflict of interest. Z.H. is the founder of PharmInVivo Ltd., Pécs, Hungary. Z.H. is stakeholder of ALGONIST Biotechnologies GmbH, Wien, Austria. The funder had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript or in the decision to publish the results. A.W. owns shares in LightOx Ltd. and Nevrargenics Ltd., companies licensed to pursue commercial applications of the compounds.

Sample Availability: Samples of the compounds are available from the authors upon suitable request.

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In Silico, *In Vitro* and *In Vivo* Pharmacodynamic Characterization of Novel Analgesic Drug Candidate Somatostatin SST_4 Receptor Agonists

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Background: Somatostatin released from the capsaicin-sensitive sensory nerves mediates analgesic and anti-inflammatory effects via its receptor subtype 4 (SST_4) without influencing endocrine functions. Therefore, SST_4 is considered to be a novel target for drug development in pain, especially chronic neuropathy which is a great unmet medical need.

Purpose and Experimental Approach: Here, we examined the *in silico* binding, SST_4-linked G protein activation and β-arrestin activation on stable SST_4 expressing cells and the effects of our novel pyrrolo-pyrimidine molecules (20, 100, 500, 1,000, 2,000 $\mu g \cdot kg^{-1}$) on partial sciatic nerve ligation-induced traumatic mononeuropathic pain model in mice.

Key Results: The novel compounds bind to the high affinity binding site of SST_4 the receptor and activate the G protein. However, unlike the reference SST_4 agonists NNC 26-9100 and J-2156, they do not induce β-arrestin activation responsible for receptor desensitization and internalization upon chronic use. They exert 65–80% maximal anti-hyperalgesic effects in the neuropathy model 1 h after a single oral administration of 100–500 $\mu g \cdot kg^{-1}$ doses.

Conclusion and Implications: The novel orally active compounds show potent and effective SST_4 receptor agonism *in vitro* and *in vivo*. All four novel ligands proved to be full agonists based on G protein activation, but failed to recruit β-arrestin. Based on their potent antinociceptive effect in the neuropathic pain model following a single oral administration, they are promising candidates for drug development.

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Abbreviations: EF1, elongation factor 1; $hsstr_4$, human somatostatin receptor subtype 4; NMRI, naval medical research institute; IRES, internal ribosomal entry site; RLUs, relative luminescence units; RMSD, root mean square deviation; SST_1-SST_5, somatostatin receptor subtypes 1–5; TM3, transmembrane domain 3; Tris–EGTA, tris-ethylene glycol bis(2-aminoethyl)tetraacetic acid.

Keywords: neuropathic pain, drug discovery, G protein coupled receptor, somatostatin, somatostatin receptor subtype 4, molecular, modeling

INTRODUCTION

Targeting somatostatin receptors as novel analgesic and anti-inflammatory drug developmental approaches has emerged after our team discovered that somatostatin was released from the activated capsaicin-sensitive peptidergic sensory nerve endings into the systemic circulation which leads to anti-inflammatory and anti-hyperalgesic actions at distant parts of the body (Pintér et al., 2006; Szolcsanyi et al., 2011; Pintér et al., 2014; Schuelert et al., 2015; Shenoy et al., 2018; Hernández et al., 2020; Kuo et al., 2020). These effects were mimicked by a synthetic heptapeptide agonist, TT-232, acting on the somatostatin receptors subtype 4 and 1 (SST_4 and SST_1) located on both primary sensory neurons and immune cells (Pintér et al., 2002; Helyes et al., 2004; Szolcsányi et al., 2004). J-2156, a highly selective and efficacious non-peptide SST_4 receptor agonist inhibited nocifensive behavior in the second phase of the formalin test, adjuvant-evoked chronic inflammatory mechanical allodynia, and sciatic nerve ligation-induced neuropathic mechanical hyperalgesia (Sándor et al., 2006). Furthermore, J-2156 decreased neuropeptide release from the peripheral terminals of peptidergic sensory neurones, as well as neurogenic and non-neurogenic acute inflammatory processes and adjuvant-induced chronic arthritic changes (Helyes et al., 2006; Elekes et al., 2008). In accordance with the above findings, in SST_4 receptor knockout mice acute and chronic inflammatory as well as neuropathic hyperalgesia were more severe than in wild types (Helyes et al., 2009). In addition to the peripheral nervous system, the SST_4 receptor is present in several central nervous system regions involved in the regulation in pain, such as the spinal cord, hippocampus and amygdala (Schreff et al., 2000; Selmer et al., 2000a; Selmer et al., 2000b). All these data provide strong proof of concept that small molecule non-peptide SST_4 receptor agonists are promising drug candidates for novel analgesic development. Furthermore, it is also important, that SST_4 does not mediate endocrine actions of somatostatin.

Based on these data, SST_4 agonists have recently become the focus of interest and development pipeline of several pharmaceutical companies for the treatment of chronic pain with one compound being tested in phase 1 clinical trial (Lilly, 2020; Stevens et al., 2020). We synthesized and patented novel pyrrolo-pyrimidine molecules (Compound 1, Compound 2, Compound 3, Compound 4) (see details in the Supplementary Materials) (Szolcsányi et al., 2019), and in the present paper we characterize their *in silico* binding, *in vitro* receptor activation and *in vivo* anti-hyperalgesic effects after single oral administration.

METHODS

In Silico Modeling Studies

Preparation of Ligand and Target Structures

Five ligand structures were built in Maestro (Schrödinger, 2017). The semi-empirical quantum chemistry program package MOPAC (Stewart, 2016) was used to minimize the raw structures with a PM7 parametrization (Stewart, 2013). The gradient norm was set to 0.001. Force calculations were applied on the energy minimized structures and the force constant matrices were positive definite. The energy-minimized structures were forwarded to docking calculations. The structure of SST_4 receptor was created by homology modeling using the active form of adrenergic β2-receptor (PDB code: 3p0g) as template. The sequence alignment was performed as in the model constructed and described by Liu and co-workers (Liu et al., 2012). Five homology models generated by Modeller program package (Stewart, 2016) were ranked related to their Discrete Optimized Protein Energy score (DOPE score) value. The first ranked model was energy-minimized and equilibrated by GROMACS 5.0.2 (Abraham et al., 2015) as described in the previous study (Liu et al., 2012). The energy-minimized receptor structure was used as a target in the docking calculations.

Docking

Docking of all ligands was performed by AutoDock 4.2.6 (Morris et al., 2009) focused on the extracellular region of the SST_4 target. In order to reduce false positive conformations, the transmembrane and intracellular target regions were not included in the docking search. Gasteiger-Marsilli partial charges were assigned to both the ligand and target atoms in AutoDock Tools (Morris et al., 2009), and united atom representation was applied for non-polar moieties. Flexibility was allowed at all active torsions of the ligand, but the target was treated rigidly. The grid maps were prepared by AutoGrid 4. The number of grid points was determined by **Eq. 1**, where L_{max} is the length of the longest ligand structure and x is the number of grid points.

$$L_{max} + 5 = 0.375x. \quad (1)$$

The docking box was centered on the extracellular region of SST_4 including 66 × 66 × 66 grid points at a 0.375 Å spacing. Lamarckian genetic algorithm was used for global search. After 10 docking runs, ligand conformations were ranked according to the corresponding calculated interaction energy values and subsequently clustered using a root mean square deviation (RMSD) tolerance of 3.5 Å between cluster members. Rank 1 was analyzed and selected as representative structure for each ligand.

G Protein Activation Assay

Membrane fractions prepared from Chinese hamster ovary (CHO) cells stably expressing the SST_4 receptor (in Tris-Ethylene glycol bis(2-aminoethyl)tetraacetic acid (Tris–EGTA) buffer (50 mM Tris–HCl, 1 mM EGTA, 3 mM $MgCl_2$, 100 mM NaCl, pH 7.4, 10 μg of protein/sample) were used for the investigations. The SSTR4 coding sequence was cloned into a

pWPTS-derived lentiviral transfer vector containing an internal ribosomal entry site (IRES) and the green fluorescent protein (GFP) gene. The SSTR4-IRES-GFP construct was driven by the EF1 promoter. HEK293 cells were used to produce the lentiviral particles, by cotransfecting the cells with the SST4 receptor coding "transfer," pMD.G "helper" and R8.91 "packaging" vectors. The culture media of HEK293 cells containing the lentiviral particles were transferred to the CHO-K1 cells. The virus particles stably transfected the CHO cells creating the stable SSTR4 expressing CHO cell line, which was used in the further experiments. Cell culture media containing the virus particles were transferred onto CHO-K1 cells. These fractions were incubated for 60 min at 30°C in the buffer containing 0.05 nM guanosine triphosphate (GTP), labeled on the gamma phosphate group with ^{35}S ([^{35}S]GTPγS) and increasing concentrations (0.1 nM–10 μM) of test compounds. 30 μM guanosine diphosphate (GDP) was added in a final volume of 500 μl. We determined the non-specific binding in the presence of 10 μM unlabelled GTPγS and total binding in the absence of test compounds. At the end of the experiment we filtered the samples through Whatman GF/B glass fiber filters using 48-well Slot Blot Manifold from Cleaver Scientific. Filters were washed with ice-cold 50 mM Tris–HCl buffer (pH 7.4) and radioactivity was measured in a β-counter (PerkinElmer Inc., Waltham, MA, United States). Test compound-induced G protein activation was given as percentage of the specific [^{35}S] GTPγS binding detected in the absence of agonists (Markovics et al., 2012).

β-Arrestin Activation Assay

In the PathHunter™ Enzyme Fragment complementation assay (DiscoverX, Fremont, CA), pCMV Mammalian cloning vector is used to drive the CHO-K1 SSTR4 cell lines to express both GPCR fused to a small enzyme donor fragment ProLink (PK), and β-Arrestin tagged with Enzyme Acceptor fragment. Upon stimulation of GPCR, β-arrestin binds to the prolink leading to the complementation of the enzyme fragments. The signal is then detected by adding the chemiluminescent reagent.

β-arrestin2 CHO-K1 SSTR4 cells were plated at a density of 20,000 cells/well in white 96 well plates and incubated overnight at 37°C. Cells were then loaded with a range of SST_4 receptor agonists' concentrations (10^{-12}–10^{-5} M) in the assay media for 90 min at 37°C. Determinations were made in duplicates. The detection reagents were added and the incubation continued at room temperature for 60 min. The agonist mediated β-arrestin 2 interaction was determined using the detection reagents according to the manufacturer's instructions. Chemiluminescence indicated as relative luminescence units (RLUs) was measured on EnSpire Alpha Plate Reader (Perkin Elmer).

Animals and Ethics

Male NMRI (named after the U.S. Naval Medical Research Institute) mice (8–12-week-old, 35–40 g weight) were used in the pain experiments. They have the highest nociceptive threshold among all mouse strains (Leo et al., 2008). Partial sciatic nerve ligation is a well-known, widely used, reproducible method to induce neuropathic pain in mice, characterized by significant allodynia and hyperalgesia, mimicking human neuropathic pain (Malmberg and Basbaum, 1998; Shields et al., 2003). We performed the first series of behavioral experiments with NMRI mice. Since we observed that the individual results show significant differences within each group including the control group, we used male C57Bl/6 mice (12–16-week-old, 25–30 g weight) for this purpose to be comparable with previous behavioral studies (Scheich et al., 2016; Scheich et al., 2017).

Mice were bred in the Laboratory Animal House of the Department of Pharmacology and Pharmacotherapy of the University of Pécs, kept in standard plastic cages at 24–25°C, under a 12–12 h light–dark cycle and provided with standard rodent chow and water *ad libitum*.

The study was designed and conducted according to European legislation (Directive 2010/63/EU) and Hungarian Government regulation (40/2013., II. 14.) on the protection of animals used for scientific purposes. The project was approved by the Animal Welfare Committee of the University of Pécs and the National Scientific Ethical Committee on Animal Experimentation of Hungary and licensed by the Government Office of Baranya County (license No. BA1/35/55-50/2017). We made all efforts to minimize the number and suffering of the animals used in this study. The group size in our experiments was chosen based upon free available power analysis program (Power and Sample Size.com, 2020) and our previous experiences using similar experimental protocols. The minimal required number for sufficient statistical power was 7. After the experiments, mice were sacrificed by cervical dislocation.

Measurement of the Mechanonociceptive Threshold of the Hindpaw and Partial Sciatic Nerve Ligation-Induced Neuropathic Pain Model of the Mouse

To measure the mechanical threshold of both hindpaws, mice were placed individually in small cages with a framed metal mesh floor. The mechanonociceptive thresholds of the mouse hindpaw were determined with the Dynamic Plantar Aesthesiometer (Ugo Basile Dynamic Plantar Aesthesiometer 37400; Comerio, Italy). This electronic von Frey device applied pressure to the plantar surface of the hindpaw with a blunt-end needle which continuously rose for 4 s until 10 g force. The force at which a paw withdrawal response occurred is registered by the equipment and it was taken as the mechanonociceptive threshold. Paw withdrawal automatically turned off the stimulus.

After conditioning, three control mechanonociceptive hindpaw threshold measurements were performed on three consecutive days. Mice were then anesthetized by the combination of ketamine (100 mg·kg^{-1}, i.p.) and xylazine (10 mg·kg^{-1}, i.p.) and placed under a dissection microscope. The right sciatic nerve was isolated from the surrounding connective tissues at a proximal site and the dorsal 1/3–1/2 of the nerve was tightly ligated with only one 8–0 silk suture in order to induce traumatic sensory mononeuropathy (Seltzer et al., 1990). The surgery was performed under aseptic conditions,

including sterile gloves, mask and sterile instruments. The animals were placed on a heating plate after the operation and monitored until complete awakening. The mechanonociceptive threshold of the plantar surface of the hindpaws was measured again on the seventh postoperative day in order to detect the development of the neuropathic pain-like state mechanical hyperalgesia in response to the nerve ligation expressed as percentage decrease compared to the mean three initial (pre-surgery) control values. Animals that failed to show at least 20% hyperalgesia were excluded from the experiment (107 out of 358 animals; 70% success rate of the operation), since they did not have obvious neuropathic pain. Subsequently, the test compounds or the vehicle methylcellulose were applied orally (in a volume of 20 ml·kg^{-1} body weight) and threshold measurements were repeated 60 min later in order to compare mechanical hyperalgesia before and after the treatment. The anti-hyperalgesic effects of the test compounds were expressed in percentage by the following formula: ((hyperalgesia before drug treatment—hyperalgesia after drug treatment)/hyperalgesia before drug treatment) · 100. The intact contralateral paws were also measured for comparison.

The experiment consisted of 15 separate series and the animals were randomized to receive the respective treatment or the vehicle. The experimenter was blinded from the treatment the animals received. The number of animals in the control group was at least four per day to minimize the bias caused by the different experimental days. Therefore, the total number of animals in the different experimental groups ranged from 7 to 19 (see details in the respective figures).

Determination of Anxiety and Spontaneous Locomotor Activity: Elevated Plus Maze (EPM) and Open Field Test (OFT)

Anxiety behavior was examined in the EPM apparatus consisting of two open and two closed arms that are extended from a common central platform. The platform was 60 cm above floor level, the floor and the walls of each arm were plastic and painted gray. Sixty min after oral administration of the vehicle or Compound 2 (500 μg·kg^{-1}), mice were placed in the center of the maze and the time they spent in the open arms during the 5-min experiment was measured (Lister, 1987; Kraeuter et al., 2019a; He et al., 2020). The surface of the maze was cleaned with 70% ethyl alcohol after each test to remove permeated odors from previous animals. There were 10 mice in each group.

Spontaneous locomotor activity and anxiety level was determined in the OFT composed of a plastic box (39 cm × 39 cm × 39 cm) with white floor and gray walls. Sixty min after the oral administration of the vehicle or Compound 2 (500 μg·kg^{-1}), mice were placed individually in the center of the box and were observed for 5 min. The arena was cleaned with 70% ethyl alcohol after each trial to remove permeated odors from previous animals (Kraeuter et al., 2019b; He et al., 2020). Behavioral parameters were recorded and analyzed by EthoVision XT 8.0 (Noldus Information Technology, Wageningen, Netherlands) motion tracking software. The number of the animals are 10 in each group.

Data and Statistical Analysis

Graphs and calculations were made using GraphPad Prism (GraphPad Prism version 8.0.1 for Windows). Curves of both G protein activation and β-arrestin 2 recruitment assays were fit by nonlinear regression using the sigmoidal dose–response equation. In the G protein activation assay we performed three experiments in triplicates. In β-arrestin assay, the experiments were conducted twice. In one experiment there were six different concentrations of each drug, each concentration was tested in duplicates to provide $n = 2$.

Results are expressed as means ± S.E.M. The maximum responses for all compounds in β-arrestin 2 recruitment assay were compared using one-way ANOVA with Dunnett's post hoc test. Data of neuropathic pain model were analyzed by one-way ANOVA Bonferroni's Multiple Comparison Test for comparing the anti-hyperalgesic effects in the different groups. Data of behavioral experiments were compared using Student's unpaired t-test except the number of rearings which were made using the Mann-Whitney U-test. The levels for statistically significant differences were set as $^{*}p < 0.05$, $^{**}p < 0.01$.

Materials

In the SST_4 receptor activation assay all the compounds were dissolved in dimethyl sulfoxide (DMSO). The concentration of the stock solutions was 10 mM, that was diluted with distilled water or assay medium to reach the final concentrations. For the *in vivo* experiments 1 mg of the compounds was suspended thoroughly in 1 ml 1.25% methylcellulose solution dissolved in sterile bidistilled water to get a 1,000 μg·ml^{-1} stock solution freshly every experimental day. Most microsuspensions looked opalescent, they were shaken properly, sonicated, and further diluted with 1.25% methylcellulose to obtain the 1, 5, 25, 50 and 100 μg·ml^{-1} solution for oral administrations (20 ml·kg^{-1} body weight for the 20, 100, 500, 1,000 and 2,000 μg·kg^{-1} dose). The solutions were shaken and sonicated again directly before use. The vehicle was always 1.25% methylcellulose dissolved in sterile bidistilled water.

Tris–HCl (PubChem CID: 93573), EGTA (PubChem CID: 6207), $MgCl_2$ (PubChem CID: 5360315), NaCl (PubChem CID: 5234): Reanal, Budapest, Hungary; GTP (PubChem CID: 135398633) : BioChemica International Inc., Melbourne, FL, United States; GDP (PubChem CID: 135398619), urea (PubChem CID: 1176), acetic acid (PubChem CID: 176): Sigma, St. Louis, MO, United States; dimethyl sulfoxide (DMSO, PubChem CID: 679): Szkarabeusz Ltd., Pécs, Hungary; [^{35}S]GTPγS: Institute of Isotopes, Budapest, Hungary; CHO-K1 cells: European Collection of Authenticated Cell Cultures (ECACC Cat# 85051005, RRID:CVCL_0214), SST_4 receptor-expressing cell line was prepared in our laboratory; methylcellulose (MC; Ph. Eur. V.; PubChem CID: 44263857): Central Pharmacy of the University of Pécs, Pécs, Hungary; $hsstr_4$ cAMP CHO-K1 (RRID:CVCL_KV83) and $hsstr_4$ β-arrestin 2 CHO-K1 cells (RRID:CVCL_KZ14): DiscoverX, Fremont, CA; methylcellulose (MC;Ph.Eur.V.; PubChem CID: 44263857): Central Pharmacy of the University of Pécs, Pécs, Hungary.

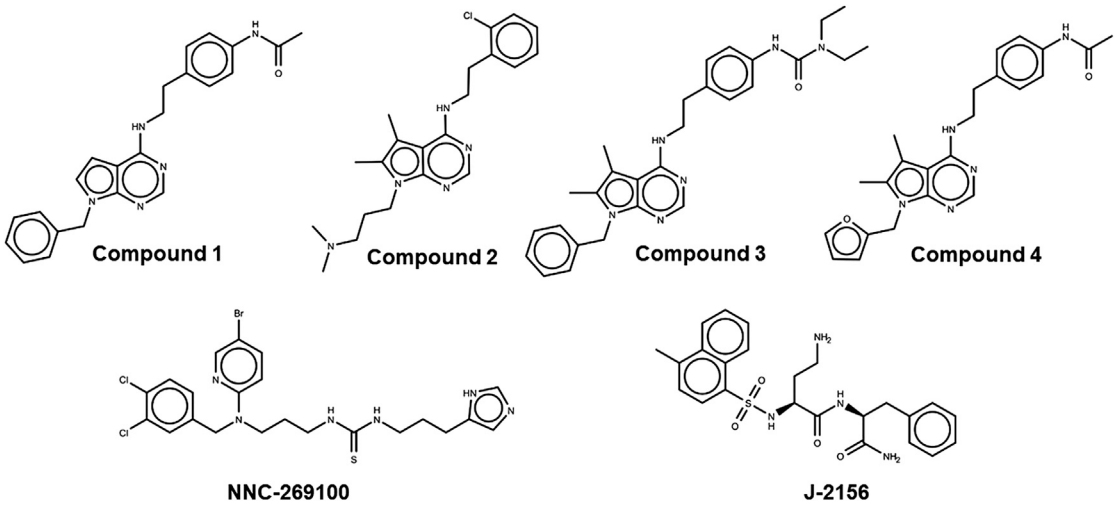

FIGURE 1 | Lewis structures of the tested new pyrrolo-pyrimidine ligands (upper panel) and the high affinity reference molecules (lower panel).

TABLE 1 | Target residues interacting with representative docked ligand structures within 3.5 Å.

	NNC-269100	J2156	Compound 1	Compound 2	Compound 3	Compound 4
Tyr18			x	x	x	
Val67	X		x	x	x	
Ser70	Xx	xx	xx	xx	xx	
Ala71				x		
Trp76	X		x	x	x	
Cys83	Xx	xx	xx		xx	xx
Arg84			x			x
Val86					x	
Leu87	Xx	xx	xx	xx	xx	
Asp90		x		x	x	x
Pro153			x			x
Asn163			x			x
Pro169	xx	xx	xx	xx	xx	xx
Ala170			x			x
Trp171	xx	xx	xx		xx	xx
His258	xx	xx				xx
Val259		x		X		
Ile262	xx	xx	xx	xx	xx	
Tyr265			X			
Fit (%)	82	82	73	73	82	45
E_{inter}	−6.58	−6.58	−8.17	−6.67	−7.97	−7.17

Amino acids interacting with the docked representatives within 3.5 Å are marked with a cross. Gray color shows the amino acids interacting with reference molecules. Double cross indicates amino acids interacting with both reference molecules.

RESULTS

In Silico Modeling and Binding Assay

Two high affinity SST_4 agonist reference compounds NNC-269100 and J-2156 (Liu et al., 1998) were used in the present study. They were shown to bind a region called high affinity binding pocket in previous studies composed of amino acids Tyr18, Val67, Ser70, Ala71, Cys83, Asp90, His258, Val259, Ile262, Leu263. Serial numbering of target residues follows that of the previous study (Liu et al., 2012). Docking of the references and four new Compounds (**Figure 1**) to the SST_4 target was performed as described in Methods. It was found that interaction energy values of the docked representatives of Compounds 1–4 do not show significant differences if compared with the high affinity reference molecules NNC-269100 and J-2156 (**Table 1**) Amino acids interacting with the representative docked ligands are marked with a cross in **Table 1**. Reference molecules have interaction with eleven target (showed with gray color in **Table 1**) residues that are identical (10^{-5} M) more than 60% (showed with double cross in

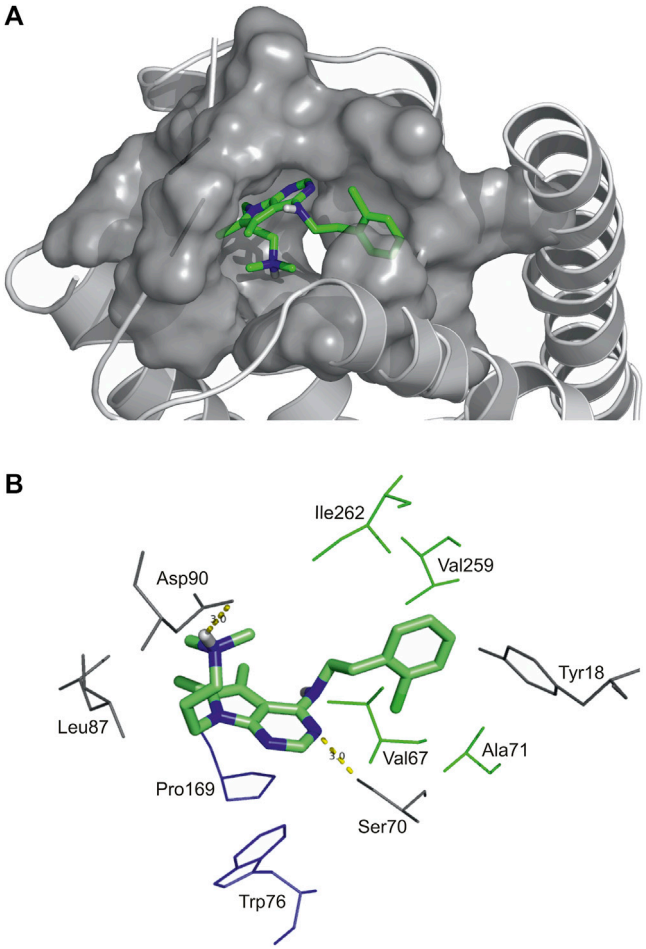

FIGURE 2 | High affinity binding pocket with Compound 2 in SST_4 receptor **(A)**, Binding pattern of Compound 2: hydrophobic pocket composed of Val67, Ala71 in TM2, Val259 and Ile262 in TM7, aromatic-aromatic interactions with Pro169 and Trp76, H-bonds (yellow) with Asp90 and Ser70 **(B)**.

Table 1). Fit % means the ratio of identical interacting residues of the references calculated for each compound. It shows that the ratio of interacting target residues for Compounds 1–3 is similar as that of the reference molecules. However, fit % of Compound 4 is 45%, it has interaction with Asp90, the key amino acid suggested essential role in ligand binding and receptor activation. Analyzes of the residues interacting with the representative docked ligand conformations within 3.5 Å showed that the new compounds maintain the contact with amino acids similarly to reference molecules, likewise to the interaction energy. Thus, reference ligands and new compounds have overlapping binding site on SST_4.

As an example, atomic details of binding of Compound 2 to SST_4 is further shown in a close-up view (**Figure 2**). The chlorobenzyl group of Compound 2 is buried in a hydrophobic pocket formed by Val67, Ala71 in TM2, Val259 and Ile262 in TM7 (TM2/TM7 hydrophobic cavity (Liu et al., 2012). The 7H-pyrrolo[2,3-d] pyrimidine core of molecule has aromatic-aromatic interactions with Pro169 and Trp76 and stabilized by a H-bond with Ser70. Furthermore, there is an ionic interaction between Asp90 on TM3 and tertiary amine group of Compound 2. It is presumed based on site-directed mutagenesis studies that an ionic interaction between Lys9 of endogenous peptide and the conserved aspartic acid on TM3 of all SST receptors has a crucial role in ligand binding and receptor activation (Kaupmann et al., 1995; Nehrung et al., 1995; Ozenberger and Hadcock, 1995; Chen et al., 1999; Liu et al., 2012).

SST_4 Receptor-Coupled G Protein Activation

Based on the *in silico* binding results, the SST_4 receptor activating potential of the new compounds was measured and compared to the reference agonist NNC 26-9100 and J-2156 (Engström et al., 2005). We found concentration-dependent stimulation in the [^{35}S]GTPγS binding assay on SST_4-expressing CHO cells (**Figure 3**.). The EC_{50} values demonstrating the potency of the ligands were, 75 , 28 , 16 and 24 nM for Compound 1, 2, 3 and 4, respectively (n=3 independent experiments with each compound). The maximal activation values over the basal activities of the receptor showing the efficacy of the compounds were 242.7 ± 26%, 213 ± 9%, 220 ± 7% and 228.7 ± 9%, in cases of Compounds 1, 2, 3 and 4, respectively. Thus, all these compounds are potent and effective SST_4 receptor agonists.

Effects of Compounds 1–4 on SST_4 Activation-Related β-Arrestin 2 Recruitment

Subsequently, we investigated the ability of the agonists to mediate β-arrestin two recruitment, measured as an increase in the chemiluminescent signal. The novel ligands displayed no detectable β-arrestin 2 recruitment in the PathHunter assay (testing range: 10^{-12}–10^{-5} M). However, the reference compounds, NNC 26-9100 and J-2156 showed marked β-arrestin 2 recruitment (**Figure 4**).

Anti-Hyperalgesic Effect of Compounds 1–4 in the Partial Sciatic Nerve Ligation-Induced Neuropathy Model

In response to the partial sciatic nerve ligation, 37.3 ± 0.8% mechanical hyperalgesia (drop of the mechanonociceptive threshold) developed on the seventh postoperative day, while the thresholds of the contralateral paws did not change compared to the baseline values. Treatment with the 500 μg·kg^{-1} oral dose of Compound 1, 2, 3 and 4 significantly increased the mechanonociceptive threshold of the treated paw 60 min later showing anti-hyperalgesic effects, while the vehicle had no effect (Compound 1: 52.1 ± 5.4% vs. vehicle: 14.7 ± 6.1%; Compound 2: 54.6 ± 13.6% vs. vehicle: 7.8 ± 8.1%; Compound 3: 57.0 ± 16.1%vs. vehicle: 12.0 ± 7.2%; Compound 4: 57.2 ± 14.6% vs. vehicle: 10.0 ± 7.6%). In case of Compound 2, the 100 μg·kg^{-1} dose also had a significant anti-hyperalgesic effect (Compound 2: 64.4 ± 14.3% vs. vehicle: 7.8 ± 8.1%). Higher doses of the compounds had smaller effects not reaching statistical significance making the dose–response relationship bell-shaped (**Figure 5**.).

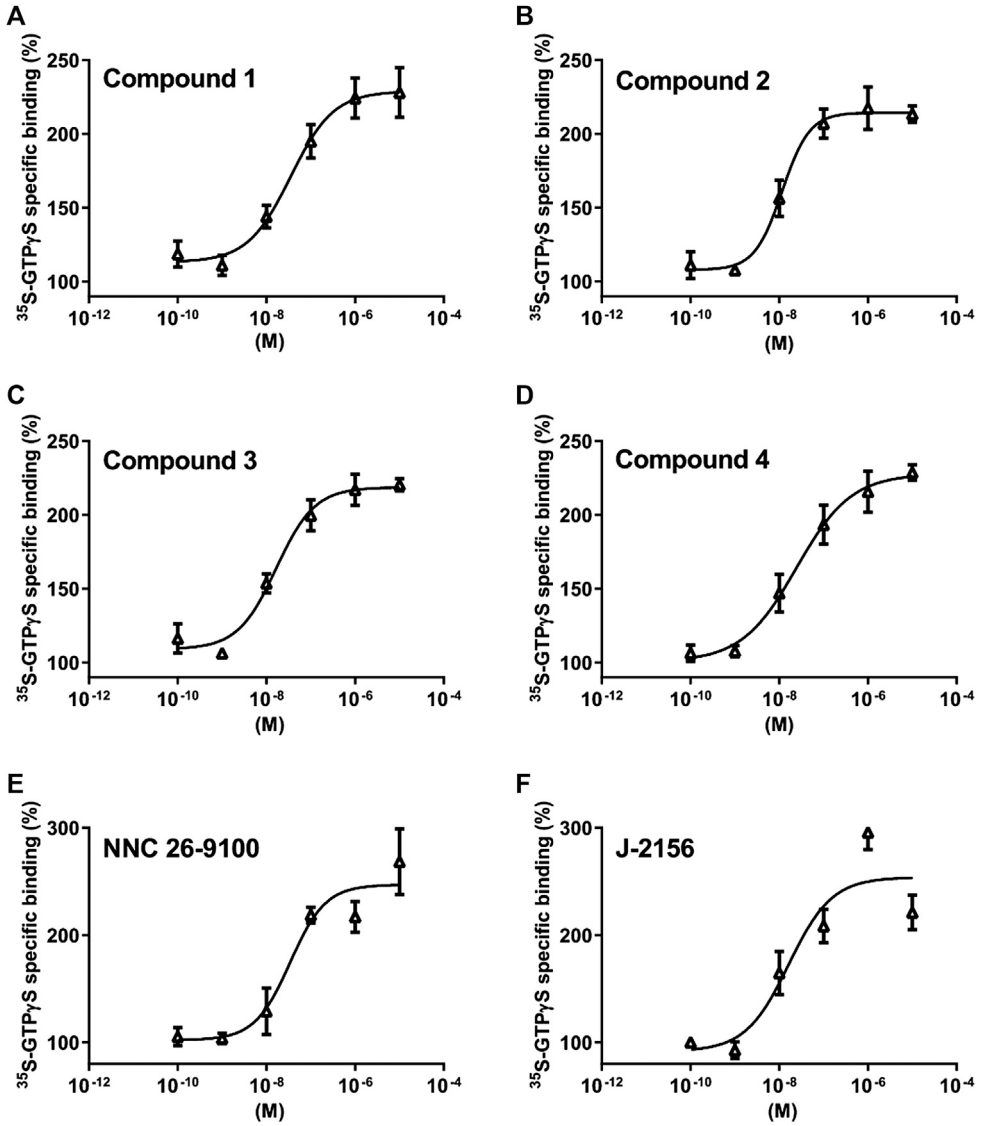

FIGURE 3 | Effect of Compounds 1–4 compared with reference molecules NNC 26-9100 and J-2156 on SST_4 receptor-linked G protein activation. [^{35}S]GTPγS binding induced by the compound in SST_4-expressing CHO cells. The ligand-stimulated [^{35}S]GTPγS binding reflects the GDP–GTP exchange reaction on α subunits of G proteins by receptor agonists. Increasing concentrations of all compounds result in similar concentration-dependent stimulations of [^{35}S]GTPγS binding. Each data point represents the mean ± SEM of n = 3 independent experiments, each performed in triplicates.

Spontaneous Locomotor Activity and Anxiety Level Are Not Influenced by Compound 2

Neither spontaneous locomotor activity nor anxiety-related behaviors in the OFT and the EPM were influenced by Compound 2.

There was no significant difference in the time spent in the open arms of the EPM (Compound 2: 52.8 ± 7.4 s vs. vehicle: 51.0 ± 8.5 s) or in the distant 1/3 of the open arms (Compound 2: 9.1 ± 3.1 s vs. vehicle: 6.2 ± 2.8) between Compound 2- and vehicle-treated mice (**Figure 6**.).

None of the parameters in the OFT, such as the distance moved (Compound 2: 1,798 ± 180.8 cm vs. vehicle: 1,824 ± 130.2 cm), velocity (Compound 2: 6.0 ± 0.6 m/s vs. vehicle: 6.1 ± 0.4 m/s), time spent moving (Compound 2: 56.0 ± 5.2 s vs. vehicle: 56.3 ± 3.7 s), time spent in center zone (Compound 2: 59.8 ± 8.7 s vs. vehicle: 59.5 ± 4.0 s), and number of rearings (Compound 2: 31.1 ± 4.1 vs. vehicle: 30.6 ± 3.2) differed significantly between Compound 2- and vehicle-treated mice (**Figure 7**.).

DISCUSSION

In the present study, four novel ligands designed for agonism at SST_4 somatostatin receptor have been characterized. *In silico*

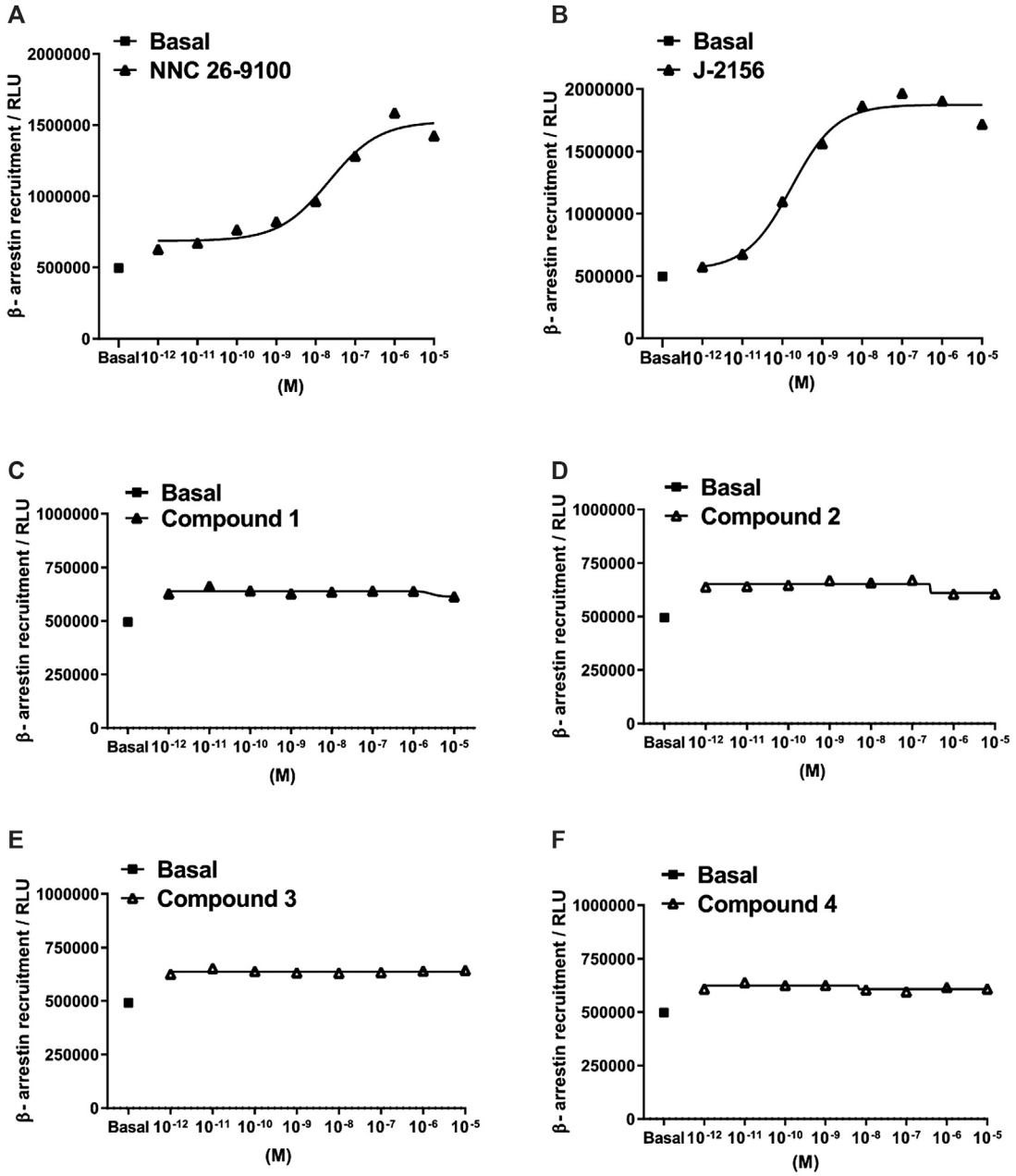

FIGURE 4 | Concentration-response curves of Compounds 1–4 in the β-arrestin 2 recruitment assay. Data represent concentration–response curves of the novel compounds expressed as relative luminescence units (RLU) in comparison to the reference compounds NNC 26-9100 and J-2156. All values are means ± SEM (n = 2 experiments). In each experiment, data points were obtained in duplicates.

modeling studies revealed that Compounds 1–4 interact with the receptor with similar energy and have overlapping binding sites on the SST_4 receptor (Liu et al., 2012) as the reference ligands NNC 26-9100 and J-2156 (**Table 1**). The binding region of J-2156 composed of amino acids Tyr18, Val67, Ser70, Ala71, Cys83, Val259 is overlapped to the binding site called high affinity binding pocket and described by Liu and coworkers (Liu et al., 2012). Docking calculations revealed that Compounds 2–4 maintain the interaction with Asp90 of TM3, as a key residue suggested by previous experimental studies with J-2156 (Kaupmann et al., 1995; Nehrung et al., 1995; Ozenberger and Hadcock, 1995; Chen et al., 1999; Liu et al., 2012). However, neither Compound 1 nor the high affinity reference NNC 26-9100 bind to the conserved aspartic acid. As they have interaction with similar residues in a high percent, our above findings suggest an alternative binding mode for these ligands.

Stimulation of G protein-coupled receptors by agonists regulates multiple downstream pathways through alpha and

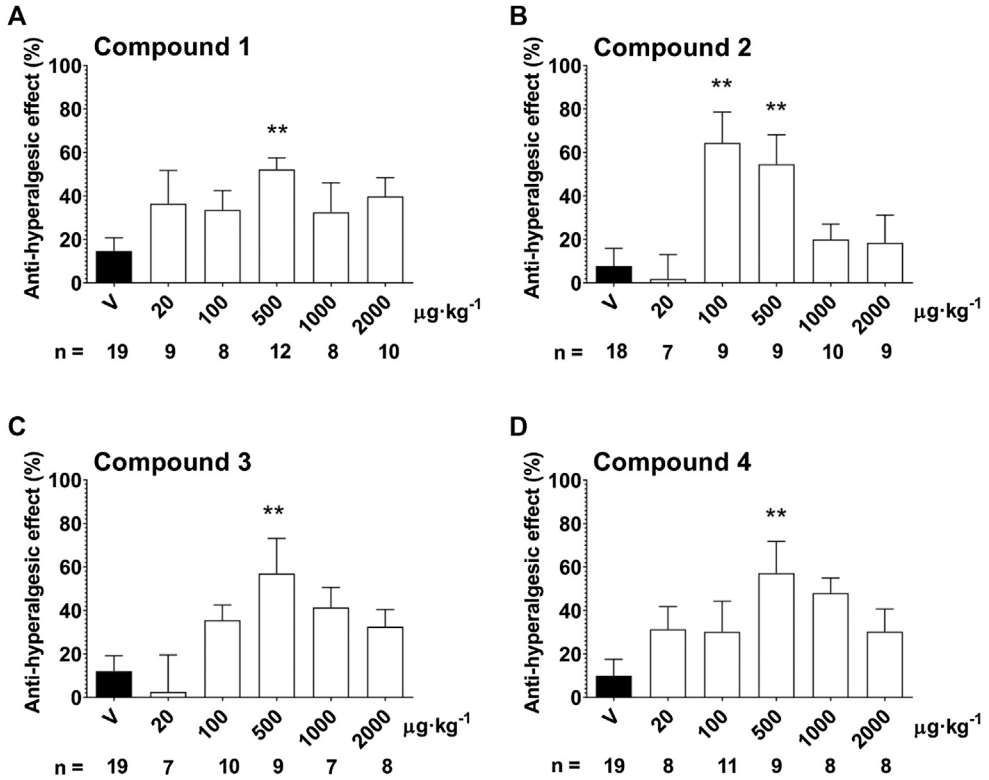

FIGURE 5 | Anti-hyperalgesic effect of a single oral treatment with Compounds 1–4 7 days after partial tight ligation of the sciatic nerve in the mouse. Columns represent anti-hyperalgesic effect 60 min after treatment with the respective test compound compared to pre-treatment control values. Each column represents the mean + S.E.M. of *n*. Data were analyzed with one-way ANOVA Bonferroni's Multiple Comparison Test (*$p < 0.05$, **$p < 0.001$ vs. vehicle control values).

beta–gamma subunits of the various G proteins. In the G protein activation assay performed on SST_4 receptor-expressing CHO cells, all the four novel compounds evoked concentration-dependent increases in [^{35}S]GTPγS binding reflecting the GDP–GTP exchange reaction on the alpha subunit of G protein similarly to the reference agonists NNC 26-9100 and J-2156. As NNC 26-9100 and J-2156 proved to be full agonists of SST_4 in a previous study (Liu et al., 1999) and the maximal achievable activation was comparable with that of the other four investigated compounds, all the novel ligands can be considered as full SST_4 agonists. Based on the EC_{50} values, the novel ligands displayed similar potencies, but Compound 3 was the most potent.

Agonist-evoked activation of heptahelical receptors also stimulates G protein-coupled receptor kinases phosphorylating the activated receptor, thereby allowing attachment of β-arrestin proteins to the receptor. While β-arrestin recruitment/binding physically obstructs the G protein coupling with the receptor, additional mechanisms have been revealed by which β-arrestins ensure efficient blockade of G protein signaling and thus, desensitization of the heptahelical receptors (Shenoy and Lefkowitz, 2011). Although β-arrestins were initially held responsible only for desensitization and down-regulation of these receptors, newer data support the view that they can also initiate several signal transduction mechanisms including e.g. activation of mitogen-activated protein kinase enzymes

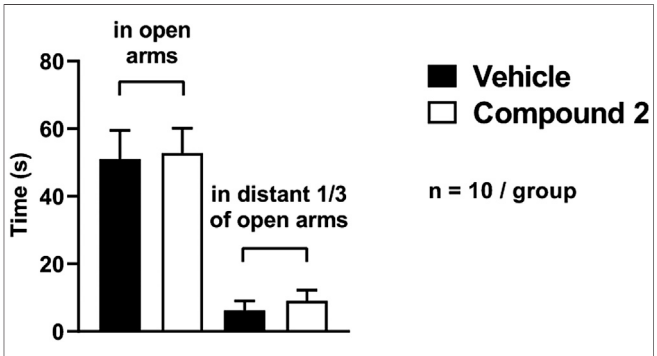

FIGURE 6 | Anxiety-like behavior quantification using EPM. Results are expressed as means ± S.E.M., data were analyzed by Student's unpaired t-test, *n* = 10/group.

(Lefkowitz, 2005). Furthermore, the existence of these two distinct pathways (i.e. G protein-dependent and β-arrestin-mediated) allows for biased agonism (also called stimulus trafficking) meaning that some ligands may act exclusively through either G protein-dependent or β-arrestin-mediated cascade (Rajagopal et al., 2010). In case of the SST_4 receptor, a dissociation of G protein activation and desensitization of a cellular effect with some agonists has been revealed, but the

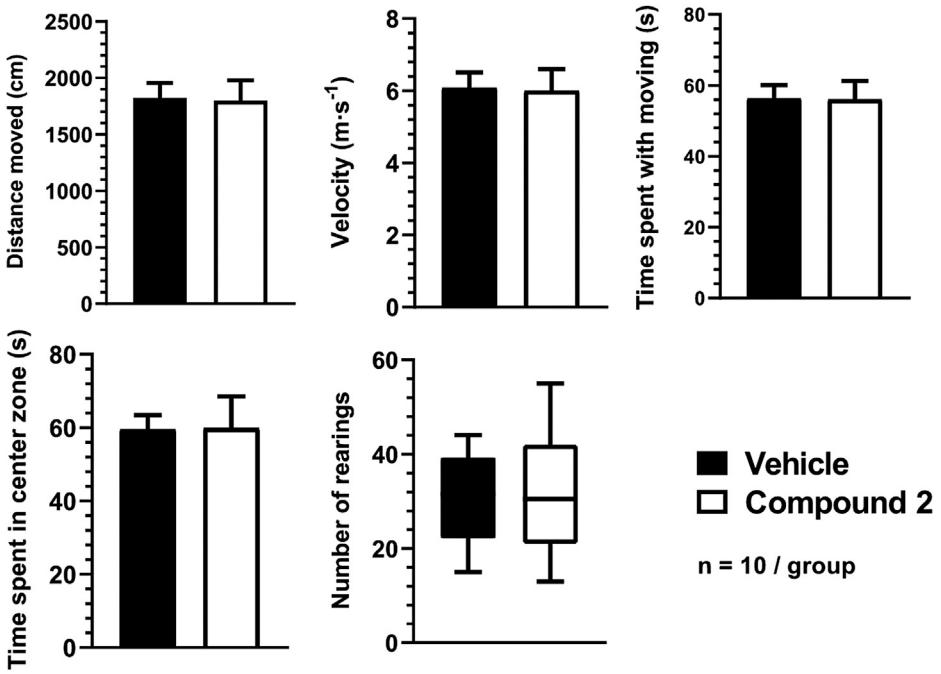

FIGURE 7 | Spontaneous locomotor activity and anxiety-like behavior quantification using OFT. The number of rearings are expressed as the geometric mean with 95% confidence intervals and statistical comparisons were made using the Mann-Whitney U-test. All other data are determined as mean ± S.E.M. and were compared using the Student's unpaired t-test, *n* = 10/group.

possible role of β-arrestins in the latter response has not been demonstrated so far (Smalley et al., 1998; Engström et al., 2006). In addition, both reference compounds showed an association with β-arrestin 2 recruitment. It was a surprising finding that all four novel SST_4 receptor agonists failed to evoke any detectable β-arrestin 2 recruitment. This result can be interpreted as biased agonism with Compounds 1–4 meaning that they initiate G_i protein-mediated receptor activation, but they are unable to recruit β-arrestin. The latter feature may be advantegous if it results in smaller degree of SST_4 desensitization to SST_4 receptor agonists upon repeated administration. However, if β-arrestin-mediated signaling also contributes to some potential therapeutic effects of SST_4 receptor agonists, this biased agonism may reduce some effects mediated by these receptors. Further studies are needed to clarify these issues. It is worth to mention that the SST_{2A} somatostatin receptor, biased agonism has also been demonstrated (Schonbrunn, 2008).

The high *in vitro* efficacy and potency of the novel SST_4 agonists made them suitable for *in vivo* testing of their antinociceptive activity. In the mouse model of traumatic neuropathic pain employing partial sciatic nerve ligation (Seltzer et al., 1990), a decrease of the mechanonociceptive threshold of the hindpaw occurred indicating the development of mechanical hyperalgesia. Following oral administration, all novel compounds were able to increase the mechanonociceptive threshold evoking anti-hyperalgesic effects. Interestingly, no conventional dose–response relationship could be established for these drugs. Bell-shaped dose–response curves could be determined for all compounds with two lower and two higher statistically ineffective doses, while the middle dose ($500\ \mu g \cdot kg^{-1}$) produced a significant anti-hyperalgesic effect. Similar efficacies corresponding to about 50–60% anti-hyperalgesic actions were observed for all drugs. Compound 2 also proved to be more potent than the other three ones as it was already effective at the $100\ \mu g \cdot kg^{-1}$ dose. The (minimal) effective anti-hyperalgesic dose of these novel SST_4 agonists is rather low indicating high *in vivo* potencies of the compounds. The reason for the bell-shaped dose–response relationship is not clear. The SST_4 receptors are present in pain-related brain regions (Kecskés et al., 2020) and also on primary sensory neurons including the peripheral terminals. We showed earlier that SST4 activation by the selective agonist J-2156 inhibits the release of sensory neuropeptides, such as substance P, calcitonin gene related peptide and somatostatin (Helyes et al., 2006). Therefore, the potential inhibitory effect of SST_4 agonists on the release of endogenous inhibitory mediators, such as somatostatin and opioid peptides cannot be excluded and might explain the lack of dose-response relationship or the bell-shaped dose-response curves. The anti-hyperalgesic effect of the compounds is not accompanied by modulated spontaneous locomotor activity and/or anxiety level, as shown by the results obtained with Compound 2, suggesting selective actions on the pain pathway.

We clearly see a significant therapeutic potential in stable, orally active, non-peptide SST_4 agonists. On the basis of the data obtained with the compounds tested in previous work as well as the present studies, these agents appear to possess broad-spectrum antinociceptive activity in models of both inflammatory and neuropathic pain (Kántás et al., 2019). Regarding the mode of action, a similarity with opioid analgesics is apparent: in both cases G_i protein-coupled, typically presynaptically/prejunctionally located receptors are activated. This

may result in—among other actions—reduction of the release of a huge array of proinflammatory and/or pronociceptive mediators from peripheral and central endings of nociceptive primary sensory neurons. This mechanism is in sharp contrast with that of receptor antagonists which can only inhibit the action of the endogenous agonist(s) of the respective receptor. As the SST_4 receptor does not appear to be involved in the myriad of endocrine effects of somatostatin (mediated by SST_2, SST_3 and SST_5 receptors), a good tolerability can be predicted for these agents. A great interest of drug companies is indicated by Lilly's recently announced licensing agreement for CNTX-0290, a SST_4 receptor agonist studied in a phase 1 clinical trial (Lilly, 2020; Stevens et al., 2020).

CONCLUSION

The novel pyrrolo-pyrimidine compounds are effective and potent SST_4 receptor agonists as shown by their *in silico* binding to the high affinity binding site and G protein activation on SST_4-expressing cells, but do not recruit β-arrestin suggesting biased agonism. They inhibit chronic neuropathic mechanical hyperalgesia following a single oral administration of a low dose (500 $\mu g \cdot kg^{-1}$), therefore, they are promising candidates for the development of a completely novel group of analgesic drugs for a huge unmet medical need.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee on Animal Research of Pécs University.

AUTHOR CONTRIBUTIONS

ZH, EP, and EB designed the study, BK, ES, RB, CH, JA, AS, LH, AgH, AdH and AK performed parts of experiments, interpreted the data and performed data analysis and BK, ES, PB, GP, EP, and ZH drafted the manuscript and revised it critically for intellectual content. All authors read and approved the final version of the manuscript before submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.601887/full#supplementary-material.

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The heptapeptide somatostatin analogue TT-232 exerts analgesic and anti-inflammatory actions via SST_4 receptor activation: In silico, in vitro and in vivo evidence in mice

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ABSTRACT

Since the conventional and adjuvant analgesics have limited effectiveness frequently accompanied by serious side effects, development of novel, potent pain killers for chronic neuropathic and inflammatory pain conditions is a big challenge. Somatostatin (SS) regulates endocrine, vascular, immune and neuronal functions, cell proliferation through 5 G_i protein-coupled receptors (SST_1-SST_5). SS released from the capsaicin-sensitive peptidergic sensory nerves mediates anti-inflammatory and antinociceptive effects without endocrine actions via SST_4. The therapeutic use of the native SS is limited by its diverse biological actions and short plasma elimination half-life. Therefore, SST_4 selective SS analogues could be promising analgesic and anti-inflammatory drug candidates with new mode of action. TT-232 is a cyclic heptapeptide showing great affinity to SST_4 and SST_1. Here, we report the in silico SST_4 receptor binding mechanism, in vitro binding (competition assay) and cAMP-decreasing effect of TT-232 in SST_4-expressing CHO cells, as well as its analgesic and anti-inflammatory actions in chronic neuropathic pain and arthritis models using wildtype and SST_4-deficient mice. TT-232 binds to SST_4 with similar interaction energy (-11.03 kcal/mol) to the superagonist J-2156, displaces somatostatin from SST_4 binding (10 nM to 30 μM) and inhibits forskolin-stimulated cAMP accumulation (EC_{50}: 371.6 ± 58.03 nmol; E_{max}: 78.63 ± 2.636 %). Its i.p. injection (100, 200 μg/kg) results in significant, 35.7 % and 50.4 %, analgesic effects upon single administration in chronic neuropathic pain and repeated injection in arthritis models in wildtype, but not in SST_4-deficient mice. These results provide evidence that the analgesic effect of TT-232 is mediated by SST_4 activation, which might open novel drug developmental potentials.

Chemical compounds

Chemical compounds studied in this article TT-232 (PubChem CID: 74053735).

1. Introduction

Somatostatin (SS) also called as somatotropin release-inhibiting factor (SRIF) was firstly described as a growth hormone (GH) inhibiting factor. It has two active forms containing 14 or 28 amino acids, due to the alternative splicing of a single preproprotein [1]. SS-14 is

Abbreviations: CHO, chinese hamster ovary; DPA, dynamic plantar esthesiometer; GH, growth hormone; SIRF, somatotropin release-inhibiting factor; SS, somatostatin; SST_{1-5}, somatostatin receptor subtype 1–5; WT, wild type; RMSD, root mean square deviation; CFA, complete Freund's adjuvant; KO, knock out.

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ordinarily found in the brain, while SS-28 in intestinal enteroendocrine cells. SS regulates endocrine functions such as inhibiting GH, prolactin, thyreotropin, insulin, glucagon, gastrin, secretin, motilin, cholecystokinin secretion, modulates vascular, immune and neuronal functions, as well as cell proliferation via 5 G_i protein-coupled receptors [2,3]. These SST_{1-5} receptors are grouped according to their agonist binding abilities: the SRIF1 group receptors bind octapeptide analogues with high affinity (SST_2, SST_3 and SST_5), while the SRIF2 ones (SST_1 and SST_4) with low affinity [4]. The SRIF2 group receptors were proposed to be crucial for the anti-inflammatory and antinociceptive effects of SS without inducing endocrine actions [5–7]. Administration of SS inhibits several pain conditions in patients, such as postoperative, cancer-related [8], and osteoarthritic pain [9]. Since 1998 our group has provided numerous data that SS originated from the activated capsaicin-sensitive peptidergic sensory nerve endings mediates anti-inflammatory and antinociceptive effects at remote parts of the body [6,10–13]. Among others, in rat models, exogen SS significantly inhibited the mustard oil-induced vasodilatation and plasma protein extravasation [13] and mechanical hyperalgesia in carragenin-induced inflammation [14]. Octreotide is a stabile SS analogue which binds with high affinity to the SST_2, SST_3, SST_5 receptors. In a double-blind study, severe headache suffered acromegalic patients were treated by single dose of octreotide that exerted rapid and permanent analgesic effect was not revoked by intravenous naloxone. Then the patients received octreotide for months without any sign of tolerance, dependence or unwanted sedative side effect [15]. A study involving patients with rheumatoid arthritis, showed that the intraarticular injection of SS-14 for 15 days decreases inflammation acute phase parameters, improves the result of telethermography and relieves the pain at rest and on the movement of the knee joint [16]. The epidural injection of SS resulted in complete postoperative pain relief of the patients who had undergone abdominal surgery and in other study octreotide i.v. administration also reduced the postoperative pain after major abdominal surgery [8,17].

However, due to their endocrine effects, neither SS nor octreotide can be applicable as analgesics. Among 5 receptors of SS, SST_2, SST_3 and SST_5 are primarly responsible for the endocrine effects, while SST_1 and SST_4 mediate the analgesic and anti-inflammatory actions [5–7,18]. SST_4 is expressed in both peripheral and the central nervous system, where it plays an important role in the pain transmission, such as in primer afferent neurons, dorsal root ganglions, dorsal horn of the spinal cord, somatosensory cortex, hippocampus, amygdala and periaqueductal grey matter [19,20]. TT-232 is a cyclic heptapeptide which was suggested to be a SST_4 / SST_1 agonist with greater affinity compared to other somatostatin receptors [21,22]. Furthermore, it is also a tyrosine-kinase inhibitor, thus it was originally developed as antitumor drug candidate with significant antiproliferative effects. It reduced the proliferation of 20 different human tumors and exerted apoptotic actions in vitro [21]. In our earlier studies TT-232 also inhibited nociceptive processes such as mechanical allodynia and exerted a wide range of anti-inflammatory effects in rat models. TT-232 in 5–20 μg/kg range, dose-dependently inhibited the partial sciatic nerve injury-induced mechano-nociceptive hyperalgesia and the carragenin-induced paw oedema in rats, but did not showed a dose–response correlation during the inhibition of bradykinin-induced plasma extravasation. In other study with rats, somatostatin and its synthetic analogues, such as TT-232 inhibited the non-neurogenic dextran oedema and the mustard oil-induced neurogenic plasma extravasation in μg/kg dose range. The subcutaneous pre-treatment of TT-232 (2 × 530 nmol/kg/day) for 18 days, significantly inhibited the Complete Freund's Adjuvant (CFA)-induced bilateral arthritis [5]. Toxic side effects were not observed at doses which reduced inflammation [5]. Subsequent studies provided data that it increases the thermal nociceptive threshold and improves diabetic neuropathic hyperalgesia at doses of 10–100 μg/kg, inhibits the nocifensive behaviour in the first and the second phase (55 % and 66 % inhibition) of formalin test at 80 μg/kg [7]. In mouse models, capsaicin-induced ear oedema was inhibited by 10 and 20 μg/kg dose of TT-232 [18] and it significantly diminishes the number of phenylquinone-induced writhes at doses of 20 and 200 μg/kg (70 % and 75 % inhibition) [7]. In contrast to native somatostatin and other analogues, TT-232 did not affect gastrin and GH secretion [23] which opens new horizons for development of SST_4 selective new analgesic agents [22,24–26].

Here, we report on the in silico modelling of the SST_4 receptor binding of TT-232 using a SST_4 receptor model generated by homology modelling, competitive binding and inhibition of cAMP in SST_4-expressing Chinese hamster ovary (CHO) cells. SST_4-mediated antinociceptive actions of chronic neuropathic and arthritic pain and the effects of TT-232 on oedema formation in CFA-induced arthritis were also investigated in mouse models.

2. Materials and methods

2.1. In silico modelling

2.1.1. Target and ligand preparation

The SST_4 receptor model was created with homology modelling approach as detailed in our previous study [27]. In the present study, the μ opioid receptor structure (PDB code: 5c1m) was used as a template for homology model construction. 10 models were built with the Modeller program package [28] and ranked by the objective function calculated by the program. The first ranked model was subjected to energy-minimization as described in the next paragraph, and used as a target structure. SST_4 receptor amino acids are numbered according to UniProt entry P31391.

The TT-232 ligand (D-Phe-c[*Cys-Tyr-D-Trp-Lys-Cys*]-Thr-NH_2) was built in Maestro [29] taking attention to the correct configuration of D-amino acids. The raw structures of the ligand and the target were equilibrated separately using a two-step energy-minimization procedure performed in AMBER99SB-ILDN force field by Gromacs program package [30]. The structures were centred in a cubic box, in which the distance was 10 Å between the box and the solute atoms. Explicit TIP3P water molecules [31] and neutralizing counter-ions (sodium or chloride) were added to the systems. Convergence thresholds were set to 10^3 kJ/mol/nm and 10 kJ/mol/nm for the steepest descent and the conjugate gradient minimization steps, respectively. The energy-minimized structures were used for docking calculations.

2.1.2. Docking calculations

The minimized and equilibrated ligand and target structures were submitted to focused docking calculations by AutoDock 4.8 [32] program package. Addition of Gasteiger-Marsilli partial charges to both the ligand and target atoms were performed by AutoDock Tools [32] and united atom representation was applied for hydrogen atoms in apolar bonds. All active torsions of the ligand were flexible, while the target was set to be rigid. The grid box was centred to target residue Asp126 that plays fundamental role in ligand bonding and receptor activation according to the literature [33–36]. The box size was set to 80x80x80 grid points with 0.375 Å spacing using AutoGrid 4. Global search was accomplished by Lamarckian genetic algorithm. After 10 docking runs, ligand conformation ranking and subsequent clustering were based on the corresponding calculated interaction energy values and a tolerance of 3.5 Å root mean square deviation (RMSD) between cluster members [37], respectively. Representative ligand structure of Rank 1 (the rank with the best interaction energy) was further analysed.

2.2. In vitro examinations

2.2.1. Competition binding analysis

CHO cells expressing SST_4 receptor were analysed in competition binding assays using TT-232 in increasing concentrations (100 nM to 300 μM). Confluent cells were used on 24-well plates. Cells were washed two times with assay buffer (5 mM KH_2PO_4 (7778–77-0), 5 mM $MgCl_2$ (7786–30-3), 10 mM HEPES (7365–45-9), 1 % (wt/vol) bovin serum

albumin (9048–46-8) and 150 mM NaCl (7647–14-5) (pH 7.4)) and incubated in the same buffer at room temperature for 30 min. Bacitracin (1 mg/ml, 1405–87-4) and [^{125}I-Tyr11]somatostatin-14 were added in 1 ml buffer for 30 min at room temperature (SS-14 (38916–34-6) was obtained from Sigma-Aldrich Ltd, Hungary, [^{125}I-Tyr11]somatostatin-14 was labelled by us using ^{125}I isotope purchased from Isotope Institute, Hungary). We washed the cells twice with ice-cold assay buffer and collected in 8 M urea/3 M acetic acid (1 ml) and measured radioactivity by a γ-counter [38]. Materials used for cell cultures and binding analysis were purchased from Sigma-Aldrich Ltd, Hungary.

2.2.2. cAMP accumulation assay

CHO-K1 cell line was maintained in Dulbecco's Modified Eagle's Medium (DMEM, Thermo Fisher Scientific, USA) supplemented with 2 mM L-glutamine (Thermo Fischer Scientific, USA, 56–85-9), 10 % fetal bovine serum (FBS), 1x penicillin/streptomycin (Thermo Fisher Scientific, USA, 61–33-6/57–92-1). Cells were cultured and kept at 37 °C, 5 % CO_2 incubator until 70–80 % confluence.

CHO-K1 cells expressing the human SST_4 receptor-expressing (Eurofins DiscoverX, Fremont, CA, USA) were cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F12, Thermo Fisher Scientific, USA) and kept at 37 °C, 5 % CO_2 incubator until 70–80 % confluence. This media was supplemented with 2 mM L-glutamine (Thermo Fischer Scientific, USA), 10 % fetal bovine serum (FBS), 1x penicillin/streptomycin (Thermo Fischer Scientific, USA), and 800 μg/ml selection antibiotic G418 (Eurofins DiscoverX, Fremont, CA, USA).

Level of cAMP was measured using the DiscoverX HitHunter™ cAMP assay kit (Eurofins DiscoverX, Fremont, CA, USA). 10 mM stock solutions were prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich Ltd, Hungary, 67–68-5) and kept at −20 °C until future use. Cells were seeded into a white 96-well assay plate in 100 μl cell plating reagent (Eurofins DiscoverX, Fremont, CA, USA) at a density of 20,000 cells/well and incubated overnight at 37 °C, 5 % CO_2. In the next day, cell plating reagent (Eurofins DiscoverX, Fremont, CA, USA) was aspirated and replaced with PBS. A series of serial dilutions (10 pmol – 10 μmol) of compounds with PBS containing the phosphodiesterase inhibitor rolipram (Sigma-Aldrich Ltd, Hungary, 61413–54-5) and the adenylate cyclase stimulator forskolin (Sigma-Aldrich Ltd, Hungary, 66575–29-9) (100 μmol) was performed. Then, cells were treated with different concentrations of the SST_4 ligands for 30 min at 37 °C, each in duplicates. Once ligand treatment was completed, each following step involved incubations with the assay reagents (Eurofins DiscoverX, Fremont, CA, USA) at room temperature. The chemiluminescent signal corresponding to the cAMP concentration was detected using a PerkinElmer EnSpire Alpha plate reader. The data were expressed as cAMP accumulation in proportion to the percentage of the forskolin response.

2.3. In vivo investigations

2.3.1. Partial sciatic nerve ligation: Traumatic mononeuropathy pain model

One conditioning and two initial measurements of mechano-nociceptive threshold were performed on three consecutive days. On the fourth day mice were anesthetized with ketamine (100 mg/kg, i.p., 6740–88-1) and xylazine (10 mg/kg, i.p., 7361–61-7). The proximal 1/3–1/2 part of the right sciatic nerve was tightly ligated with one 8–0 silk suture under a dissection microscope to evoke traumatic sensory mononeuropathy [39]. During the operation animals were placed on a heating blanket and monitored until complete awakening. The mechano-nociceptive threshold of the hindpaws was determined again on the 7th postoperative day to demonstrate the mechanical hyperalgesia (decrease of the mechano-nociceptive threshold in response to the nerve ligation expressed as percentage compared to the mean pre-surgery values). Animals not showing at least 20 % mechanical hyperalgesia were excluded, since they did not show significant neuropathic pain behaviour.

Mechano-nociceptive thresholds of the hindpaws were investigated by Dynamic Plantar Aesthesiometry (DPA, Ugo Basile Dynamic Plantar Aesthesiometer 37400; Comerio, Italy). This is an electronic device with a blunt-end needle, which evolves pressure to the plantar surface of the hindpaw through the metal mesh floor of the cages. The maximal of 10 g force is reached in 4 s. The equipment automatically turns off the stimulus when mice withdrawal response occurs and registers the mechano-nociceptive threshold.

In the in vivo experiments 1 mg/ml stock solution was made from the TT-232 (Tocris Bioscience, Cat. No. 4639) with acetate buffer (pH 3.5) freshly every experimental day. Further dilutions were made with phosphate buffer (PBS - pH 7.3) to get 10 and 20 μg/ml solution (10 ml/kg body weight for the 100, and 200 μg/kg dose). The vehicle was always the mixture of acetate and phosphate buffer.

The TT-232 (in 100 or 200 μg/kg doses) or the vehicle were given intraperitoneally and threshold measurements were repeated 30 min later to compare pre- and post-treatment mechanical hyperalgesia. The analgesic effect of the TT-232 was expressed in percentage as described earlier: ((hyperalgesia before drug treatment—hyperalgesia after drug treatment)/hyperalgesia before drug treatment) · 100 [40].

2.3.2. Chronic arthritis model

Arthritis was evoked by intraplantar injection of 50 μl of Complete Freund's Adjuvant (CFA, killed Mycobacteria in paraffin oil, 1 mg/ml; Sigma, St. Louis, MO) into the right hindpaw and s.c. into the root of the tail. The s.c. injection was repeated on the next day into the tail to achive systemic effects and to show more similarities with the human disease [41–43]. The mechano-nociceptive threshold of the hindpaw was determined by DPA, similarly to neuropathy model, before and 4, 6, 8, 11, 13, 15, 18, 20, 21 days after CFA administration. The TT-232 (in 100 μg/kg dose) or the vehicle were applied i.p. (in a volume of 10 ml/kg body weight) every measuring day 30 min before the measurements. Mechanical hyperalgesia was expressed as % of control mechano-nociceptive threshold compared to the initial values [41,43–45]. The paw volume was determined by plethysmometry (Ugo Basile Plethysmometer 7140, Comerio, Italy) [43,45]. Volumes were measured before and 4, 6, 8, 11, 13, 15, 18, 20 and 21 days after CFA-injection. Oedema was expressed in percentage compared to the initial values [41,42].

2.3.3. Animals and ethics

Male C57Bl/6J based SST_4 receptor gene deficient (KO) [46] and wild type C57Bl/6J (WT) mice (8–12 weeks old) were bred and kept in the Laboratory Animal House of the Department of Pharmacology and Pharmacotherapy of the University of Pecs in standard polycarbonate cages under a 12–12 h light–dark cycle, at 24–25 °C and provided with standard rodent chow and water ad libitum. SST_4 knockout mice were generated by pairing heterozygote animals donated by the research group of Dr. Pierce C. Empson (Laboratory of Molecular Neuroscience, The Babraham Institute, Babraham Research Campus, Babraham, Cambridge CB22 3AT, United Kingdom). The genotype of their offsprings was identified by PCR analysis [46]. Experimental procedures complied with the recommendations of the 1998/XXVIII Act of the Hungarian Parliament on Animal Protection and Consideration Decree of Scientific Procedures of Animal Experiments (63/2010) and were approved by the Ethics Committee on Animal Research of Pecs University according to the Ethical Codex of Animal Experiments (license No. BA1/35/55–50/2017).

2.4. Statistical analysis

Graphs and calculations were made using GraphPad Prism version 8.0.1 statistical software. Results are expressed as means ± S.E.M. The number of the animals were n = 5–6/group and n = 9–16/group in CFA-induced arthritis and sciatic nerve ligation-evoked neuropathy model, respectively.

The cAMP levels were normalized considering forskolin response as

100 %. Curves were fit by nonlinear regression using the sigmoidal dose–response equation.

Data were tested by Shapiro-Wilk normality test and showed normal distribution. Baseline values of the WT and KO groups were compared with unpaired *t*-test, the mechano-nociceptive thresholds before and after the treatment with TT-232 of vehicle were compared with one paired *t*-test. The analgesic effect of the TT-232 was calculated from the mechano-nociceptive thresholds compared to the results of the control group using two-sample t-tests.

Mechanical hyperalgesia- and paw oedema induced by CFA were evaluated by two-way analysis of variance (ANOVA) followed by Bonferroni posttest.

When comparing the results of the respective groups, *p < 0.05; **p < 0.01; ***p < 0.001 and ****p < 0.0001 were considered to be significant.

3. Results

3.1. Binding of TT-232 to the SST_4 receptor determined by in silico modelling

Docking results showed that TT-232 fits to a deep binding pocket of the SST_4 receptor accessible from the extracellular region. The pocket is located in a crevice between TM3-7 helices and ECL2-3 loops. The low negative interaction energy between the representative structure of TT-232 and SST_4 receptor was −11.03 kcal/mol. The SST_4 receptor residues interacting with TT-232 (within 3.5 Å heavy atom distance threshold) are listed in Table 1. TT-232 is stabilized mainly by H-bonds in the binding pocket (Table 1, Fig. 1.), other non-covalent interactions have lower importance. The Lys5 of TT-232 resembles to residue of Lys9 of SS, the endogen ligand that plays a critical role in receptor binding and activation from the aspect of the ligand.

3.2. TT-232 displaces somatostatin from the SST_4 receptor binding

The binding ability of TT-232 (100 nM to 300 μM) on the SST_4 receptor expressed in CHO cells was determined by competition binding assay using [^{125}I-Tyr11]SS-14. TT-232 displaced the labelled SS from the SST_4-expressing CHO cells by concentration-dependent manner. The maximal value of the displacement was 94 ± 5.2 % after the administration 100 μM TT-232 (13 964 count per minute value decreased to 838). The IC_{50} value was 21.5 μM (Fig. 2.).

Table 1
The list of interacting residues and H-bridges in the complex of SST_4 and TT-232.

Ligand residues	Target residues within 3.5 Å distance from the corresponding ligand residue	H-bond between the target and the ligand residues
Phe1	Asn282	
Tyr3	Val278	
	Gln279	
	Asn282	X
Trp4	Asn199	
	Leu200	
Lys5	Leu123	
	Asp126	X
	Tyr301	X
Cys6	Phe211	
Thr7	Asp126	X
	Gly127	
	Met130	
	Phe131	
	Phe211	
	Thr215	X

3.3. TT-232 decreases intracellular cAMP concentration via SST_4 receptors

TT-232 showed a robust concentration-dependent inhibitory effect on the forskolin-stimulated cAMP production in SST_4-expressing CHO cells with similar efficacy, but lower potency as the selective SST_4 agonist reference compound J-2156. The E_{max} values for TT-232 and J-2156 were 78.63 ± 2.636 %, and 90.53 ± 1.776 %, the EC_{50} values were 371.6 ± 58.03 nmol, and 681.4 ± 63.91 pmol, respectively (Fig. 3A). Meanwhile, neither compound had any effect in CHO cells not expressing the SST_4 receptor (Fig. 3B.).

3.4. TT-232 inhibits neuropathic hyperalgesia via SST_4 receptor activation in the mouse

In the partial sciatic nerve ligation model, the mechano-nociceptive threshold dropped with approximately 37 % on the seventh post-operative day, while the thresholds of the contralateral paws remained unchanged compared to the baseline values. In case of WT mice the treatment with the 100 and 200 μg/kg dose of TT-232 significantly reduced the drop of the mechano-nociceptive threshold of the treated paw 30 min later with the following values: TT-232: 6.77 ± 0.30 g; 7.23 ± 0.29 g (24.9 ± 3.4 %; 19.6 ± 3.2 %), while the vehicle had no effect (5.57 ± 0.23 g 38.2 ± 2.3 %) (Fig. 4A). In contrast with the WT mice, TT-232 did not influence the mechano-nociceptive threshold in the $SSTR_4$-gene deficient mice. The corresponding values were the following in the 100 and 200 μg/kg dose of TT-232 or vehicle-treated groups: TT-232: 5.31 ± 0.16 g; 5.14 ± 0.15 g (34.2 ± 1.9 %; 35.77 ± 1.4 %) vs vehicle: 5.32 ± 0.11 g (35.3 ± 1.75 %) (Fig. 4B).

Analgesic effect was calculated from the changes of the mechano-nociceptive threshold. TT-232 showed 35.7 ± 8.3 % and 50.4 ± 8.4 % analgesic effect in 100 and 200 μg/kg doses in WT mice (Fig. 4C, D).

3.5. TT-232 inhibits chronic arthritic hyperalgesia via SST_4 receptor activation

Initial mechano-nociceptive thresholds of WT (7.78 ± 0.10 g) and SST_4 KO (8.09 ± 0.08 g) animals did not differ (p = 0.1102; F = 1.51) at the beginning of the experiment. CFA injection induced an approximately 30 % drop of the threshold (mechanical hyperalgesia) on the treated paw in both groups by the fourth day (saline-treated WT: 5.66 ± 0.26 g, TT-232-treated WT: 5.72 ± 0.32 g; saline-treated SST_4 KO: 5.20 ± 0.27 g, TT-232-treated SST_4 KO: 5.59 ± 0.14 g), which was stably maintained during the 21-day experimental period. TT-232 treatment (100 μg/kg, i.p. 30 min before the measurements) significantly reduced this chronic inflammatory mechanical hyperalgesia from day 6 (saline-treated WT: 5.35 ± 0.16 g, TT-232-treated WT: 6.86 ± 0.39 g), and almost abolished from day 11 (saline-treated WT: 5.91 ± 0.25 g, TT-232-treated WT: 7.4 ± 0.17 g) in WTs (Fig. 5A), but not in the SST_4 KOs (saline-treated SST4 KO: 5.62 ± 0.30 g, TT-232-treated SST4 KO: 5.72 ± 0.14 g) (Fig. 5B). Control paw volumes of WT (0.15 ± 0.003 cm^3) and SST_4 KO (0.15 ± 0.002 cm^3) animals were also similar (p = 0.68; F = 1.85). CFA injection evoked remarkable, about 100 % paw oedema by day 8 (saline-treated WT: 0.31 ± 0.02 cm^3, saline-treated SST4 KO: 0.29 ± 0.01 cm^3), which persisted throughout the experiment in all groups, it was not affected by TT-232 treatment in either WT (0.29 ± 0,007 cm^3) (Fig. 5C) or SST_4 KO mice (0.30 ± 0.01 cm^3) (Fig. 5D).

4. Discussion

In the present study we demonstrated the SST_4 receptor binding and activation of the heptapeptide somatostatin analogue TT-232 by in silico and in vitro methods, as well as its SST_4-mediated anti-nociceptive effects in chronic inflammatory and neuropathic pain mouse models.

In silico modelling revealed that TT-232 interacts with the SST_4 receptor with similar or stronger binding strength than the selective SST_4

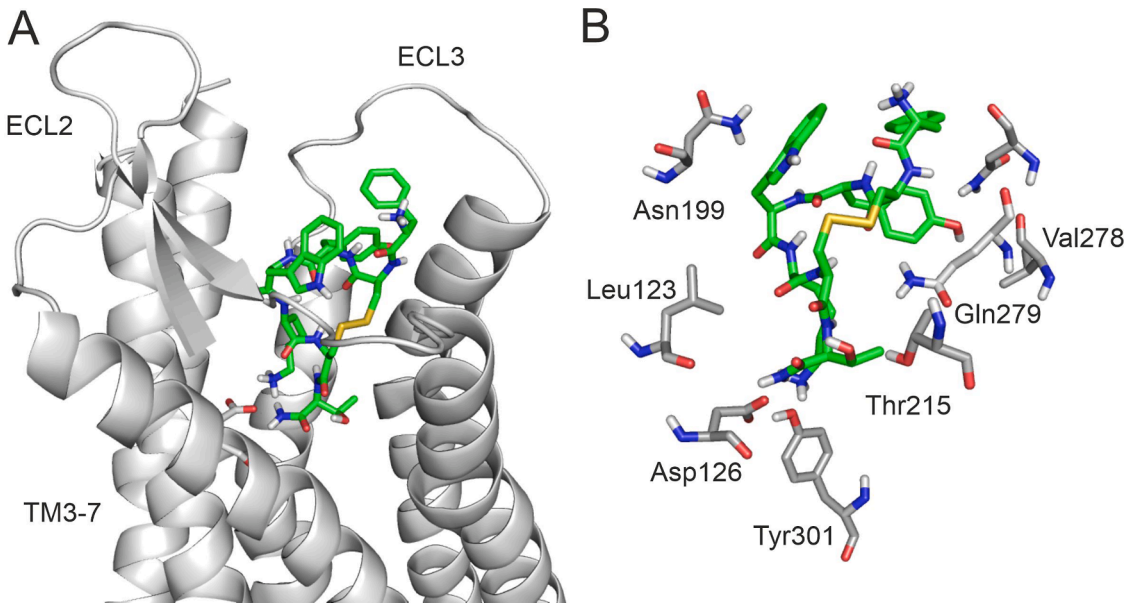

Fig. 1. A) A global view of SST_4 receptor (grey, cartoon representation) in complex with TT-232 (Rank 1 representative, green sticks) B) The close-up view of TT-232 (Rank 1 representative) in the binding pocket of SST_4 receptor. Target residues interacting with the ligand within 3.5 Å and TT-232 are colored by grey and green, respectively.

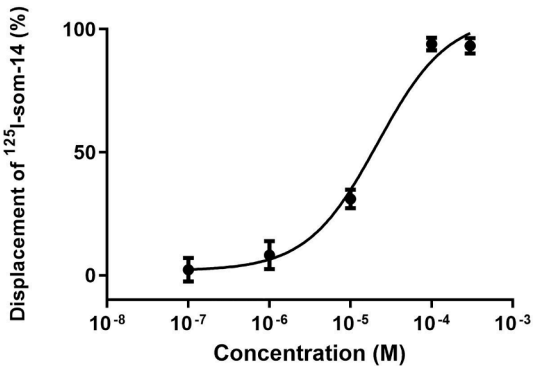

Fig. 2. Concentration-dependent displacement of [^{125}I-Tyr11]SS-14 binding from SST_4 receptors by TT-232 (100 nM-300 μM) on SST_4-expressing CHO cells. Each data point represents the mean ± SEM of n = 4 experiments.

agonist reference compounds J-2156 and NNC-269100 [40] and some novel pyrrolo-pyrimidine molecules [27]. The Lys5 of the TT-232 creates H-bonds with the conserved Asp126 of SST_4 receptor that is essential for the receptor activation proved by several experimental studies [33–36], furthermore, Lys5 is stabilized by a further H-bond with Tyr301 as well. The Lys5 of TT-232 resembles the residue of Lys9 of somatostatin, the endogen ligand that plays a critical role in receptor binding and activation from the aspect of the ligand. The intramolecular cyclization of TT-232 results in a relatively constrained structure with low conformational freedom for the peptide backbone [47] allowing rotations only at the side-chains. When previously studying the SST_1 binding ability of TT-232 it was shown that all conformations of this molecule have almost the same backbone structure in aqueous solution resulting in high inner stability [47]. Thus, the pre-formed cyclic conformation of TT-232 allows a favourably low entropy loss during binding to the SST_4 receptor. At the same time, the new interactions with Tyr301 provide a considerably large enthalpic contribution to the overall binding strength.

The in vitro binding ability of TT-232 to the SST_4 receptor confirmed the in silico results. The displacement of the radiolabelled SS by TT-232

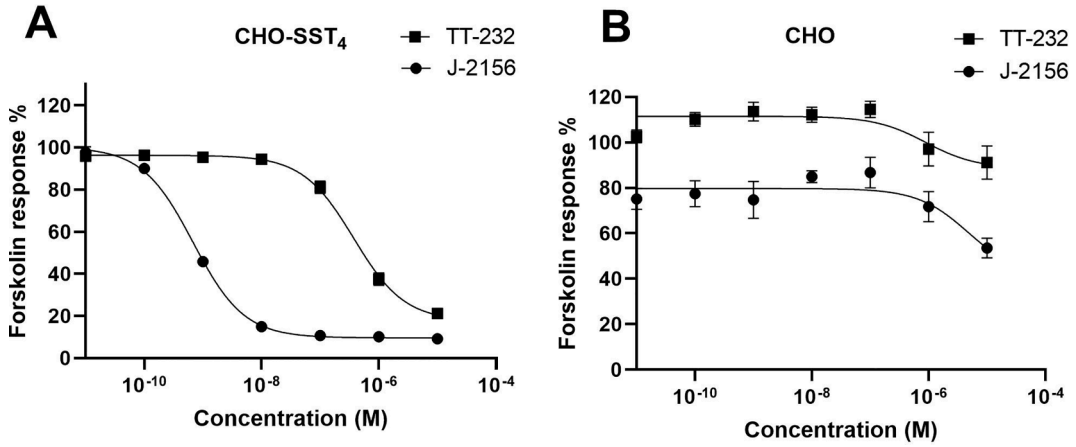

Fig. 3. Concentration-response curves of TT-232 showing cAMP accumulation levels in (A) SST_4 expressing CHO and (B) CHO cells in comparison to J-2156. All values are means ± SEM of n = (A) 10, (B) n = 8 for TT-232 and 4 for J-2156.

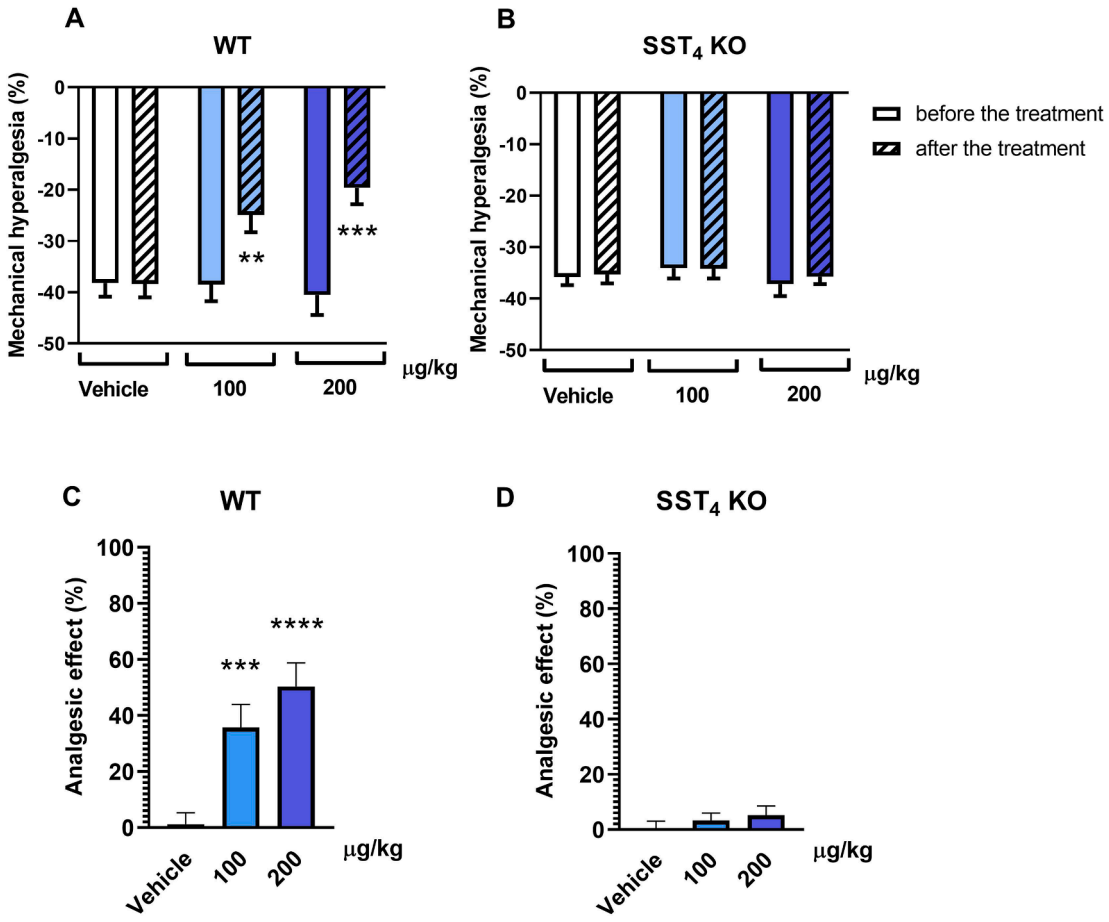

Fig. 4. The changes of the mechano-nociceptive thresholds (A, B) and analgesic effect (C, D) of TT-232 in WT (A, C) and SST_4 KO (B, D) mice. The data were compared with paired *t*-test and represented in mean ± SEM format (*p < 0,05; **p < 0,01 and ***p < 0,001 vs before the treatment, n = 9–16/group).

on SST_4 receptor-expressing CHO cells was concentration dependent and it was almost 95 % at 100 µM concentration in the competition binding assay. After proving the receptor-binding to the SST_4 receptor, we measured the intracellular cAMP level. Since SST_4 is a G_i protein-coupled receptor, it inhibits adenylate cyclase and consequent cAMP formation. Forskolin is a cell-permeable diterpene that directly activates adenylate cyclase, therefore raises the intracellular cAMP level [48]. TT-232 exerted robust concentration-dependent inhibitory effect on the forskolin-stimulated cAMP production in SST_4-expressing CHO cells similarly to the reference compound J-2156 [49], but there was no effect in control CHO cells demonstrating specific SST_4-dependent action. TT-232 has similar efficacy, but lower potency than the reference compound J-2156 in the cAMP assay.

Our previous studies have provided substantial evidence that TT-232 reduces neurogenic and non-neurogenic inflammatory processes in rats and mice [5,18], furthermore the acute and chronic airway inflammation in rats [50]. Therefore, in the present experiments its effects on neuropathic and chronic inflammatory pain conditions were investigated. Partial sciatic nerve ligation [39] is a widely used well established method to model traumatic neuropathic pain in rodents. Following the operation, significant nerve damage develops leading to abnormal sensory functions without disabling motor functions [39,51]. Both examined single administered doses (100, 200 µg/kg, i.p.) of TT-232 were able to increase the mechano-nociceptive threshold evoking dose-dependent analgesic effects on the 7th postoperative day when the neuropathic pain was fully developed. This is consistent with previous data showing that both TT-232 and the non-peptide superagonist J-2156 exerted analgesic effects (10–100 µg/kg i.p.) in rat and mouse models, TT-232 also reversed mechanical hyperalgesia [18,52].

In the adjuvant-induced chronic arthritis model, daily injections of TT-232 also inhibited mechanical hyperalgesia, but did not affect oedema formation. However, in our earlier studies, in different acute inflammation models, TT-232 (5, 10, 20 µg/kg i.v. or s.c.) significantly and dose-dependently reduced the bradykinin-, carragenin- and capsaicin-induced oedema in rats [18]. The moderate variability of the effective doses of TT-232 in the different animal models could be explained by the strain differences (rat and mouse) and the distinct pathomechanisms of the examined processes.

Since the inhibitory actions of TT-232 on hyperalgesia were not observed in SST_4-deficient mice in either the neuropathy or the arthritis model, it is suggested that its analgesic actions are mediated by SST_4 activation. This hypothesis can also be supported by our previous results related to J-2156, the selective SST_4 superagonist, which did not relieve the carragenin-evoked mechanical hyperalgesia [46]. Furthermore, increased inflammatory reactions were developed both in the oxazolone-induced allergic contact dermatitis and the endotoxin-evoked airway inflammation model in SST_4 gene deficient mice compared to the WT counterparts [46].

Original compounds with new mechanisms of action are needed for the adequate treatment of neuropathic pain conditions, since the conventional analgesics, such as NSAIDs and opioids show only limited effectiveness. The widely used adjuvant analgesics, such as amitriptyline and gabapentin only partly diminish the symptoms and often exert serious side effects. Amitriptyline, a tricyclic antidepressant, used to treat neuropathic pain caused by shingles, often causes anticholinergic side effects (obstipation, dry mouth, vomiting), palpitation, blurring of vision, drowsiness, dizziness and weight gain. Gabapentin, has been developed as an antiepileptic drug, is also used for peripheral

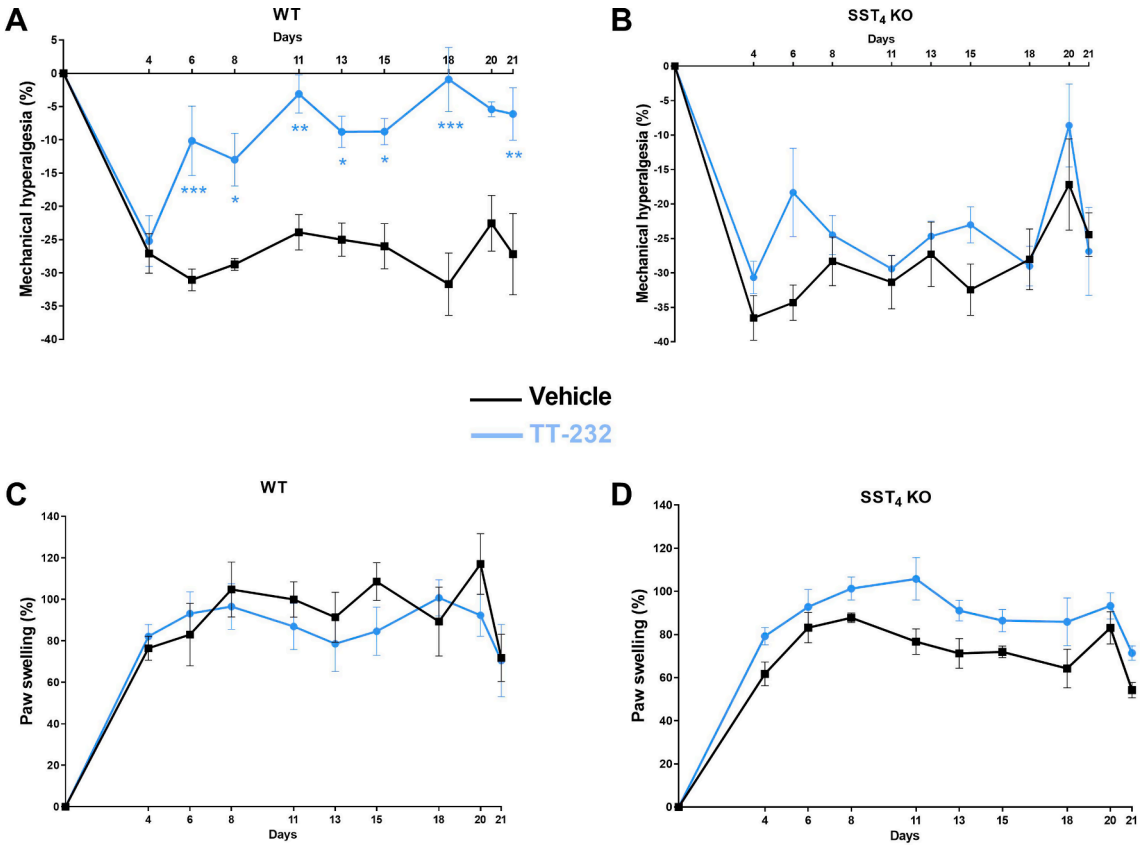

Fig. 5. Changes of the (A, B) mechano-nociceptive thresholds and (C, D) paw volumes over the 21-day investigation period after intraplantar and tail root injection of complete Freund's adjuvant in TT-232-treated (A, C) WT and (B, D) SST_4 KO mice compared to the saline vehicle-treated group. Each data point represents the mean ± SEM of n = 5–6/group (two-way ANOVA; *p < 0,05; **p < 0,01 and ***p < 0,001 vs control group).

neuropathy. It frequently causes somnolence, dizziness, ataxia, vomiting, as well as, diarrhea [53–55]. Since the SST_4 receptor selective agonists have completely different molecular mode of action than NSAIDs, opioids and adjuvant analgesics they might have reduced, more tolerable side effect spectrum. Furthermore, SST4 selective agonists compared to the non-selective somatostatin analogues do not exert endocrine actions. Octreotide and lanreotide commonly cause GI-related adverse effects such as diarrhea, nausea and abdominal pain [56,57], increased gallstone formation [58]. Exocrine pancreatic insufficiency is a common but under-recognized adverse effect induced by octreotide via inhibiting the production of cholecystokinin and secretin [59,60]. Elevated blood glucose levels occurred in case of all marketed somatostatin analogues [61,62].

In summary the present results strongly support that SST_4 could be a promising therapeutic target in the therapy of neuropathic and inflammatory chronic pain conditions. These data clearly show the effectiveness of TT-232 in chronic pain models via SST_4 receptor. Although its peptide characteristics prevent oral administration, alternative formulations, such as subcutaneous or intravenous injections, as well as nasal spray might be applicable.

CRediT authorship contribution statement

Rita Börzsei: Methodology, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Éva Borbély:** Methodology, Formal analysis, Investigation, Writing – review & editing, Visualization, Funding acquisition. **Boglárka Kántás:** Methodology, Formal analysis, Investigation, Writing – original draft, Visualization. **Lina Hudhud:** Methodology, Investigation, Writing – original draft, Visualization. **Ádám Horváth:** Methodology, Formal analysis, Investigation. **Éva Szőke:** Methodology, Investigation, Writing – original draft, Visualization, Supervision, Project administration, Funding acquisition. **Csaba Hetényi:** Conceptualization, Writing – original draft, Supervision, Project administration, Funding acquisition. **Zsuzsanna Helyes:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Erika Pintér:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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