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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General introduction

1. Pain, neurogenic inflammation and the capsaicin-sensitive peptidergic nerves

"Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage'' [1]. Therefore, it is an important alert and defense mechanism, but pain that last longer becomes chronic and negatively affect patients' quality of life [2]. Pain can be nociceptive pain that is usually associated with inflammatory diseases such as in arthritis [3], or neuropathic pain, which is associated with nerve damage such as diabetes [4]. Inflammation is the response of the body to harmful stimuli, such as infection and tissue damage that is important to restore the homeostasis. Persistent inflmmation causes chronic painful conditions and chronic inflammatory diseases [5]. The complexity of the inflammatory response is mediated by a broad range of cell types including immune cells, neurones and other non-neuronal cells [6].

Pain as stimuli activates a subdivision of peripheral nociceptive sensory neurons involved in inflammation and are characterized as ''capsaicin-sensitive afferents" as they respond to capsaicin the pungent ingredient in chili peppers [7]. This response occurs through the transient receptor potential vanilloid 1 (TRPV1). TRPV1 can also be activated by various physical and chemical stimuli, such as temperatures over 43°C, acidic extracellular conditions (pH <6), and vanilloids [8]. In addition, it was found that these capsaicin-sensitive afferents can be triggered by the transient receptor potential ankyrin 1 (TRPA1) ion channels which found to be co-expressed with TRPV1 [9]. When activated, TRPV1 induces the cellular influx of Ca^{2+} and Na^{+} ions, leading to depolarization of the nerve terminal and the formation of an action potential. Besides nociception, capsaicin-sensitive sensory nerve endings induce the so called ''neurogenic inflammation'' mediated by pro-inflammatory neuropeptides such as tachykinins and calcitonin gene-related peptide causing vasodilatation and plasma extravasation. These neurons can also release neuropeptide such as somatostatin, which exert local interaction with the inflammatory mediators and can cause systemic anti-inflammatory actions, a phenomenon that is described as the "sensocrine".

Current therapies including nonsteroidal anti-inflammatory drugs, opioids, adjuvant analgesics such as anticonvulsants and antidepressants, and topical analgesics such as lidocaine

and capsaicin have limited efficacy and cause a variety of side effects. Therefore, there is an immense need for the development of novel drugs with different mechanism of actions.

2. TRPA1 and TRPV1 channels in pain and cancer

TRPA1 and TRPV1 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 [12]. TRPV1 is highly expressed in nociceptors, hence its role in neuropathic and inflammatory pain had received intense interest among the TRP channels. In addition, TRPA1 has been found to mediate inflammatory mechanical hyperalgesia as well as cold hyperalgesia under inflammatory conditions [13], [14].

Several studies have reported the expression of TRPA1 and TRPV1 channels in non-neuronal cells such as in synoviocytes, chondrocytes, lung epithelial cells, and smooth muscle cells [15], [16]. 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 [17]. TRPA1 is highly expressed in tumors such as pancreatic adenocarcinoma, nasopharyngeal carcinoma, and prostate cancer-associated fibroblast cell cultures.

It is well-established that both TRPA1 and TRPV1 activation induces the cellular influx of Ca^{2+}. This activates Ca^{2+}-dependent signaling pathways such as the mitogen-activated protein kinase/extracellular signal regulated kinase and phosphatidylinositol 3-kinase/Akt Protein Kinase B pathways, all of which play an important role in cancer cell proliferation, migration, invasion, and apoptosis.

3. Somatostatin receptor 4

Somatostatin, also known as somatotropin release-inhibiting factor is a neuropeptide that is expressed throughout the body in its active forms of 14 to 28 amino acids and has multiple biological effects in different cells and organs. Somatostatin exerts its effect via its own membrane-bound inhibitory Gi -coupled receptors (SST_1, SST_2, SST_3, SST_4, SST_5). SST_4 is the main receptor mediating the antinociceptive and anti-inflammatory actions without endocrine effects. Activation of somatostatin receptor 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 [18].

Our research group showed that somatostatin is released from the activated capsaicin-sensitive sensory nerve endings into the systemic circulation where it can exert anti-inflammatory and anti-hyperalgesic actions [7], [19]. Both SST_2 and SST_4 are expressed in pain signaling pathways and involved in somatostatin-induced analgesia [20]. Nevertheless, activation of SST_2 is associated with several side effects such as inhibiting growth hormone release [21], [22] and insulin secretion [23]. Many studies have reported the significance of SST_4 as a potential target for novel anti-inflammatory and analgesic therapy without endocrine actions. However, somatostatin has a very short half-life, it is not suitable for drug developmental purposes. Therefore, there is a great efforts to develop a selective SST_4 agonist with a longer half-life.

4. Aims

This dissertation includes three different studies where commonly used *in vitro* cell-based assays were applied to examine and 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* as preliminary study for future investigations of their ability to activate TRPV1 and/or TRPA1 channels.

II. Testing the expression and function of TRPA1, TRPV1 in mouse and human osteosarcoma tissues and osteosarcoma K7M2 cell line 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 Chinese hamster ovary cells

1. Introduction

All-trans-retinoic acid (ATRA), the active metabolite of vitamin A, has an important role in cell differentiation, proliferation, and embryonic development. Therefore, it is used either alone or as maintenance therapy in the treatment of various skin diseases and as an anti-cancer drug. However, ATRA is prone to isomerization and oxidation, which may affect its activity and selectivity. More stable synthetic derivatives are needed to overcome these problems and offer potential valuable alternatives as pharmaceutical agents with lower toxicity.

The severe side effect profile of retinoids has limited their clinical use, particularly their systemic administration. In addition, retinoids have been studied for their genotoxicity. Different retinoids, including 13-cis-retinoic acid, caused sister chromatid exchanges in human diploid fibroblasts [24]. In addition, retinol induced chromosomal aberrations in human lymphocytes [25] , and caused DNA single strand breaks, fragmentation, and oxidative DNA damage in cultured Sertoli cells [26], [27]. ATRA and a steroidal analog EA-4 induced double strand DNA fragmentation in C2C12 mouse cells and HL-60 human acute myeloid leukemia cells [28].

Novel diphenylacetylene-based ATRA derivatives with several structural variations were synthesized in order to increase the stability. DC360 is a fluorescence when activated by visible light, DC324 is a non-active fluorescent retinoid [29]. EC23, DC525, DC540, DC645 and DC712 have an increased receptor binding ability and bioactivity [29]–[31]. These synthetic derivatives have a great potential as therapeutics for a variety of cancers and neurodegenerative diseases including amyotrophic lateral sclerosis [32].

2. Methods

2.1 Cell culturing

2.2 ATP cell-viability assay

2.3 Comet assay (Single cell gel electrophoresis)

2.4 Statistical analysis and data presentation

3. Results

Cytotoxicity may lead to false positive results in some genotoxicity assays. When a compound is tested at high concentrations, a non-genotoxic agent may cause DNA fragmentation and cell death, thus, in our study, a non-cytotoxic concentration was chosen to test the direct effect of the diphenylacetylene-based retinoids on DNA damage. At the tested (1 μM, and 10 μM) concentrations, ATRA and the synthetic analogues did not decrease cell viability, except for DC324, which caused statistically significant decrease at 10 μM. However, the viability-inhibition was only 13%, which is less than the generally accepted guidelines for the comet assay that 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.

Our study also shows that all synthetic ATRA derivatives caused DNA damaging effects similar to ATRA, but the most hydrophobic compounds, DC324, DC360 and EC23 were more toxic than ATRA, especially at the higher concentrations. These compounds have high log P structures, therefore, they may exhibit stronger off-target interactions with other proteins and DNA species. DC360 provoked the strongest genotoxic effect compared to the others, which can be explained by the presence of dihydroquinoline hydrophobic region that could also be reactive towards cellular components under certain circumstances.

4. 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. We believe that these compounds demonstrate a retinoid receptor-independent genotoxicity and that should be considered in later development and applications. Further studies are needed to identify the underlying molecular mechanisms and elucidate the complex biological activities of these compounds. It is well observed that topical application of retinoids often leads to severe local irritation, itching and stinging [33], [34]. In addition, systemically administered retinoids also cause bone pain, and severe headache [33], [35]. Recent studies have reported that retinoids selectively sensitized the TRPV1 ion channel and produced sensory hypersensitivity [36]. Furthermore, the selective RAR receptor antagonist

LE135 activated both TRPA1 and TRPV1 channels and induced pain-related behaviors [37]. 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 [38]. Therefore, 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.

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

1. Introduction

Osteosarcoma is the most common primary malignant bone tumor that is heterogeneous and inevitably linked to its microenvironment, which mediates tumor cells survival and growth [39]. Despite the improvements in research and clinical management, the survival rates for osteosarcoma patients with metastasis still below 20% [39], [40]. New agents that can suppress the growth and increase survival of osteosarcoma cells are needed to offer therapeutical benefits.

Several studies indicated that both TRPV1 and TRPA1 channels play an important role in tumor progression. However, no studies to date have identified the functional expression and/or intracellular localization of TRPA1 in osteosarcoma cancer. In addition, TRPV1 has been implicated in the regulation of cancer cell proliferation, migration, and invasion, but there is not enough data regarding functional expression and localization of TRPV1 in osteosarcoma.

2. Methods

1.1 Cell culturing, mouse osteosarcoma model, human osteosarcoma samples
1.2 RNA isolation and polymerase chain reaction (PCR) gel electropherisis
1.3 Tissue collection, and sample and cells preparation for RNAscope study
1.4 RNAscope
1.5 Radioactive ${}_{45}Ca^{2+}$ uptake experiments on K7M2 cells
1.6 ATP cell-viability assay
1.7 Statistical analysis and data presentation

3. Results

We found that AITC induces Ca^{2+} influx in K7M2 cells and decreased their viability in a dose-dependent manner demonstrating the functional expression of TRPA1 where its activation-induced effect is suggested to be via Ca^{2+}-mediated mechanisms. The TRPA1 antagonist HC-030031 partially reversed both the Ca^{2+} influx and the reduction of cell viability. 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. Our results showed that AITC could inhibit osteosarcoma cell survival through TRPA1-dependent pathway.

We also found that 48-hour capsaicin treatment caused a significant concentration-dependent loss of viability in K7M2 cells. However, capsaicin did not have any effect on Ca^{2+} influx. The TRPV1 antagonist capsazepine was not able to abolish the capsaicin-induced reduction in cell viability. These data suggest that capsaicin exerts its cytotoxic effect on these cells through TRPV1-independent pathways.

4. Conclusion

We provide here the first data regarding the functional TRPA1 expression in human and mouse osteosarcoma and potential of this channel to decrease cancer cell viability. We also demonstrate that TRPV1 expression in osteosarcoma support earlier findings suggesting cytotoxic effects of capsaicin on human osteosarcoma cells. However, we only investigated the receptor functionality in the mouse K7M2 cell line that do not have all the osteosarcoma characteristics, therefore, it is difficult to strongly conclude that TRPA1 agonists could have potential therapeutic applications. However, osteosarcoma is an extremely malignant tumor with resistance to therapy and poor prognosis, thus modulating TRPA1 might be a promising molecular tool for further broader investigations.

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

1. Introduction

Our research group showed the effects of the synthetic heptapeptide agonist TT-232 via SST_4 receptor activation. TT-232 binds SST_1 and SST_4 that are located on both immune cells and primary sensory neurons [11], [41]. Intravenous administration of TT-232 showed anti-nociceptive action that was not observed with octreotide [10]. Besides that, TT-232 decreased mechanical hyperalgesia in diabetic neuropathy [42] and arthritis models [11]. J-2156, a known potent and selective non-peptide SST_4 superagonist suppressed the nocifensive behavior in the second phase of the formalin test, sciatic nerve ligation-induced neuropathic mechanical hyperalgesia, and adjuvant-evoked chronic inflammatory mechanical allodynia [43]. J-2156 also reduced the release of pro-inflammatory neuropeptide from the peptidergic sensory nerve endings and attenuated the neurogenic and non-neurogenic inflammation in chronic arthritic model [44], [45]. Moreover, SST_4 knockout mice showed more severe neuropathic hyperalgesia and inflammatory pain compared to the wild types [46]. Therefore, SST_4 is considered as a target of interest for the development of novel drug candidates for the treatment of inflammation and chronic pain.

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 1-4) and demonstrated their interaction with the amino acids of the high affinity binding pocket similar to J-2156 and were effective in G-protein activation assay. In addition, a single oral-low dose administration these compounds inhibited chronic neuropathic mechanical hyperalgesia in mice [47]–[49]. In this dissertation, we demonstrate the binding data of these compounds to SST_4 by means of inhibiting cAMP levels in CHO cells expressing SST_4 by testing our novel pyrrolo-pyrimidine compounds comparing their effect to the reference agonists J-2156 and TT-232. We also tested the potential selective SST_4 activation by ruling out the effect on CHO-SST_2 cells.

2. Methods

2.1 Cell culturing and treatments

2.2 cAMP accumulation assay

2.3 Statistical analysis and data presentation

3. Results

J-2156 and TT-232 showed a dose-response inhibition of cAMP production in SST_4-expressing CHO cells 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 cAMP on SST_4-expressing CHO cells. In addition, cAMP inhibition was not observed with any reference or tested compounds on SST_2-expressing CHO cells demonstrating their selectivity to SST_4.

4. Conclusion

We successfully built and validated the SST_4 receptor activation by cAMP *in vitro* assay, which can be further performed in our laboratories for screening novel potential SST_4 agonists as analgesic and anti-inflammatory candidates without side effects mediated by other SST receptors. 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 further ligands testing.

Our novel pyrrolo-pyrimidine compounds can control neuropathic and inflammatory pain, but the exact molecular mechanism and the possible signaling pathways need further investigations.

Summary of the new results

- We first investigated the novel diphenylacetylene-based ATRA derivatives on cell viability and genotoxicity in 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 is no cytotoxic effect of these compounds, however, all compounds induce DNA migration. DC360 is the most toxic agent, which can be due to the high Log P structure. Our results show RAR- or RXR-independent genotoxicity, thus testing the activation of TRPV1 and TRPA1 channels as a potential cellular mechanism for this effect and/or for retinoids-related irritations and painful side effects is considered in our current and future research.

- We investigated the functional expression of TRPA1 in human osteosarcoma tissues, mouse osteosarcoma tissues and cell line. TRPA1 is expressed in human and mouse tissues as well as in mouse osteosarcoma K7M2 cells. The TRPA1 agonist AITC induces radioactive calcium uptake and reduces cell viability. This effect is blocked by the TRPA1 antagonist HC-030031. TRPA1 and TRPV1 are co-expressed in both neuronal and non-neuronal cells, where they can affect each other's function. Therefore, we investigated the TRPV1 expression in these osteosarcoma models. TRPV1 shows expression in human and mouse tissues but very low expression in K7M2 cells, this can be explained by the differences in tumor microenvironment between tissues and cell lines. Capsaicin does not induce significant calcium uptake but decreased K7M2 cell viability and the TRPV1 antagonist capsazepine does not block this effect. These findings suggest a reduction in osteosarcoma cell growth and survival by TRPA1-dependent and TRPV1–independent pathways. Different apoptosis assays are planned to be performed to reveal the potential molecular mechanisms behind these effects.

- We confirmed the SST_4 receptor as a promising target for the development of anti-inflammatory and analgesic drugs. Both J-2156 and TT-232 inhibits cAMP levels, however, the pyrrolo-pyrimidine compounds don’t show the same effect. This suggest that they may induce their anti-inflammatory and analgesic effects through different signaling

pathway. The exact 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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