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Investigation of Selected Piperazine-2,5-Diones on Their Effect on Cartilage-Related Cells †

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Abstract: Various chronic inflammatory diseases have become a problem, especially in the Western world. Whether it is inflammation of visceral organs, joints, bones, etc., it is always a physiological reaction of the body, which always tries to eradicate harmful substances and restore tissue homeostasis. Unfortunately, prolonged or chronic inflammation often results in damage to the affected tissues. Cartilage damage, diseases such as osteoarthritis, rheumatoid arthritis and arthrosis, are very common. In addition to suppressing inflammation in the joints and around the cartilage, it is advantageous to administer compounds that are capable of stimulating cartilage growth and regenerate damaged tissue. Variously substituted piperazine-2,5-dione derivatives were investigated as compounds with a potential effect on cartilage regeneration. A series of assays were performed to evaluate their cytotoxicity, anti-inflammatory activity, ability to potentiate chondrocyte proliferation, and suppress synovial cell growth. The compounds proved to be completely non-toxic for all used types of cells up to the concentration of 20 μM. Unfortunately, their evaluated biological activity proved to be insignificant in comparison with untreated cells.

Keywords: piperazine-2,5-diones; cytotoxicity; viability assay; anti-inflammatory activity; chondrocytes; synovial cells

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1. Introduction

Degenerative diseases of the bones and joints affect millions of people. Fractures of the hands, hips, spine caused by osteoporosis are associated with significant morbidity and mortality. Destruction and deformity of the joints and other complications caused by arthritis not only make movement difficult, but reduce the ability to perform routine activities, resulting in an overall reduced quality of life for patients, among other things [1–5].

Many different treatment approaches are being developed for the burning problem of increasingly common musculoskeletal degenerative diseases. Treatment options for musculoskeletal disorders are non-pharmacological, pharmacological and surgical. These are incurable diseases for this moment, so the goal of treatment is to achieve remission or low activity of the disease. The longer the treatment is started, the worse the results are and the irreversible damage to the joints. Non-pharmacological treatment is based on regular exercise (weight reduction, physical activity), rehabilitation, manipulation therapy to strengthen muscles, maintain maximum mobility and joint functionality. In advanced stages of the disease, some damaged joints can be surgically removed and replaced with

artificial implants (endoprostheses of the hip, knee, shoulder, elbow, wrist and finger joints). Surgical treatment of the patient also relieves pain in the affected joint. Pharmacological treatment includes two basic groups of drugs, which are usually combined: drugs that reduce inflammation and pain, and drugs that reduce the progression of structural damage, i.e. inhibit the destruction of articular cartilage and induce the balance of its metabolism. Non-steroidal anti-inflammatory drugs and paracetamol are used to reduce inflammation and pain. In case of acute inflammation, glucocorticoids can be given. Conventional synthetic (e.g., methotrexate), targeted synthetic JAK kinase inhibitors or biologicals (antibodies) are used as antirheumatics. In this context, it is necessary to mention that there are also many dietary supplements on the market that are intended to prevent or alleviate diseases of the musculoskeletal system. Agents that inhibit the destruction of articular cartilage are so-called chondroprotectives. Currently recommended are glucosamine sulfate and chondroitin sulfate, hyaluronic acid, avocado-soybean unsaponifiables, diacerein, *Boswellia serrata* extract, curcumin, *S*-adenosyl methionine, methylsulfonylmethane, rose hip. Alternatively, fish liver oil, omega-3 fatty acids, vitamins A, C and E in combination, vitamin K, vitamin D, ginger and collagen/gelatin are listed as beneficial dietary supplements [6–13].

Long-term administration of most of the above-mentioned drugs has negative effects on other organs, so in accordance with the concept of polypharmacology and multi-target drugs, efforts have been made to design agents that have the ability to regenerate both cartilage and bone while exhibiting anti-inflammatory activity. In addition, these agents must be non-toxic in order to be administered to long-term chronically ill patients [14–16]. Alaptide ((*S*)-8-methyl-6,9-diazaspiro-[4.5]decan-7,10-dione) was chosen as a model molecule, which in previous studies showed high regenerative abilities on the skin and mucosa and no chronic toxicity. In addition, this compound exhibits other remarkable biological properties. Alaptide was prepared at the Research Institute for Pharmacy and Biochemistry in Prague in the former Czechoslovakia in the 1980s [17–23]. The disadvantage of alaptide is its practical insolubility, so more soluble simple derivatives were designed and prepared and all compounds have been evaluated using a set of *in vitro* assays for the required biological activities.

2. Results and Discussion

The structures of all the investigated compounds are listed in Table 1 together with their biological activities. The preparation of the compounds was described previously [19,23].

Table 1. Structure and values of viability of THP-1, SW982, and primary porcine chondrocytes (Chondr.) assays [IC_{50} (μM) after 72 h incubation] of investigated compounds—alaptide (**1**) and its derivatives **2**–**5**.

Comp.	R^1	R^2	R^3	Tox IC_{50} [μM] (72 h)		
				THP-1	**SW982**	**Chondr.**
1	–$(CH_2)_4$–		–CH_3	>20	>30	>30
2	–H	–H	–H	>20	>30	>30
3	–H	–H	–CH_3	>20	>30	>30
4	–CH_3	–H	–CH_3	>20	>30	>30
5	–CH_3	–CH_3	–CH_3	>20	>30	>30

THP-1 = human monocytic leukemia; SW982 = human synovial cell line; Chondr. = primary porcine chondrocytes.

The basic safety profile of the test compounds was evaluated on the base of determined relative cell viability of different cell types related to cartilage tissue (monocytes THP-1, synovial cells SW982, and primary porcine chondrocytes). Neither alaptide (**1**) nor other piperazine-2,5-dione derivatives **2**–**5** significantly influenced cell viability, which was still 90–120% when cells were incubated for 72 h with the highest concentrations of them (20 μM for THP-1, and 30 μM for SW982 and chondrocytes). It indicates any cytotoxic effect of test molecules, on the other hand, any pro-proliferative action was observed as well. Similar, non-toxic, effect was observed for the same compounds tested on human skin fibroblast cells (BJ), a T-lymphoblastic leukemia cell line CEM, and a breast adenocarcinoma cell line MCF7 up to the concentration of 50 μM [23].

To determine an anti-inflammatory potential of the test compounds, their effect on lipopolysaccharide (LPS)-stimulated activation of NF-κB, one of the key pro-inflammatory transcription factors, was evaluated. In this assay, any of used agents was able to reduce the NF-κB activity in the concentration of 10 μM.

All obtained results from used assays showed that test alaptide (**1**) and its derivatives **2**–**5** are not able to influence the pathological features of rheumatoid arthritis. On the other hand, they possess very low cytotoxic effect on different cell types and thus they are safe for further biological experiments.

3. Experimental

3.1. Synthesis

Described piperazine-2,5-diones were characterized by Pokorna et al. [23].

3.2. Cell Lines Culture

Human synovial cell line SW982 and human monocytic leukemia THP-1 (both cell lines from ATCC, Manassas, VA, USA) were routinely cultivated in RPMI 1640 medium with stabile glutamine supplemented with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin mixture (all from Merck, St. Louis, MO, USA). Cells were passaged twice a week and their viability was regularly controlled by Trypan Blue staining.

3.3. Primary Porcine Chondrocytes Isolation

The cartilage tissue was obtained from porcine elbow joint from slaughtered pigs in a local slaughterhouse. Approximately 300 mg of tissue was twice washed in sterile phosphate buffered saline (PBS; Merck) and cut by a scalpel to ca. 1 mm^3 pieces. Cartilage pieces were covered by the solution of collagenase I (Merck) 6 mg/mL in DMEM/F12 medium (Biosera, Nuaille, France) and incubated at 37 °C for 2 h until all parts were completely lysed. After that, the enzyme was inactivated by adding of DMEM/F12 medium containing 10% FBS and 1% penicillin/streptomycin mixture. The cell suspension was filtrated through 70 μm nylon membrane and centrifuge 5 min at 150 g. Then, the supernatant was discarded, cells were resuspended in a fresh medium, counted using the Trypan Blue dye, and split into cultivation plates coated by collagen I (Corning; Kennebunk, ME, USA) in the density of $5\times10^3/cm^2$. Cells were incubated for 5 days at 37 °C in humidify atmosphere with 5% CO_2. After this period, the medium was exchanged and cells were ready for further experiments.

3.4. Cell Viability Determination

To determine cell viability, the Cell Counting Kit 8 (CCK-8; Merck) was used according to the manufacturer’s instruction. All experiments were performed in the complete cultivation medium containing 10% FBS. SW982 cells were split into 96-well plate in the concentration of 1×10^4 cells per well and let to attach overnight. Then, the medium was exchanged. THP-1 cells were seeded in the concentration of 5×10^4 cells per well. Primary chondrocytes were used after 5 day attachment as described above. When cells were prepared, they were treated by the test compounds dissolved in dimethyl sulfoxide (DMSO)

and the relative cell viability (the ratio between cells treated with compounds and cells treated with DMSO only) was measured after 72 h, as we described previously [24].

3.5. NF-κB Activity Determination

The ability of test compounds to inhibit the transcription factor NF-κB, one of the key pro-inflammatory intracellular regulator, was evaluated on THP-1 Blue NF-κB cell line (Invivogen; San Diego, CA, USA), as we described previously [25]. NF-κB was activated by lipopolysaccharide (LPS) from *E. coli* 0111:B4 (Merck) dissolved in serum-free RPMI 1640 medium (1 g/mL) after 1 h pre-treatment by test compounds dissolved in DMSO in the concentration of 10 μM.

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