chemistry proceedings

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Proceeding Paper

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

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† Presented at the 25th International Electronic Conference on Synthetic Organic Chemistry, 15–30 November 2021; Available online: https://ecsoc-25.sciforum.net/.

Abstract: Various chronic inflammatory diseases have become a problem, especially in the Western world. Whether it concerns 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. Diseases such as osteoarthritis, rheumatoid arthritis, and arthrosis, as well as cartilage damage, 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 regenerating 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, and 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 based on the comparison with untreated cells.

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

Citation: Jampilek, J.; Hosek, J.; Bobal, P. Investigation of the Effect of Selected Piperazine-2,5-Diones on Cartilage-Related Cells. *Chem. Proc.* **2022**, *8*, 108. https://doi.org/10.3390/ecsoc-25-11650

Academic Editor: Julio A. Seijas

Published: 13 November 2021

1. Introduction

Degenerative diseases of the bones and joints affect millions of people. Fractures of the hands, hips, and 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 takes to start, the worse the results, including irreversible damage to the joints. Non-pharmacological treatment is based on regular exercise (weight reduction and physical activity), rehabilitation, and manipulation therapy to strengthen muscles and maintain maximum mobility and joint functionality. In the 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). The surgical treatment of the patient also relieves pain in the affected

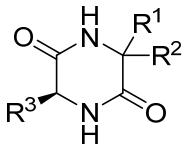
joint. Pharmacological treatment includes two basic groups of drugs, which are usually combined: drugs that reduce inflammation and pain, and drugs that reduce the progression of structural damage, i.e., inhibit the destruction of articular cartilage and induce the balance of its metabolism. Non-steroidal 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, chondroitin sulfate, hyaluronic acid, avocado-soybean unsaponifiables, diacerein, *Boswellia serrata* extract, curcumin, *S*-adenosyl methionine, methylsulfonylmethane, and 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].

The long-term administration of most of the above-mentioned drugs has negative effects on other organs; thus, 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 showed high regenerative abilities on the skin and mucosa and no chronic toxicity in previous studies. 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]. A disadvantage of alaptide is its practical insolubility, so more soluble simple derivatives were designed and prepared, and all compounds were 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 procedure 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 tested compounds was evaluated based on the 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 the compounds (20 μM for THP-1 and 30 μM for SW982 and chondrocytes). The tested molecules did not show any cytotoxic effect or pro-proliferative action. Similarly, no toxic effect was observed for the same compounds tested up to the concentration of 50 μM on human skin fibroblast cells (BJ), a T-lymphoblastic leukemia cell line CEM, and a breast adenocarcinoma cell line MCF7 [23].

To determine the anti-inflammatory potential of the tested compounds, their effect on the lipopolysaccharide (LPS)-stimulated activation of NF-κB, one of the key pro-inflammatory transcription factors, was evaluated. In this assay, the used agents were not able to reduce the NF-κB activity in the concentration of 10 μM.

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

3. Experimental

3.1. Synthesis

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

3.2. Cell Lines Culture

Human synovial SW982 and human monocytic leukemia THP-1 cell lines (both from ATCC, Manassas, VA, USA) were routinely cultivated in RPMI 1640 medium with glutamine supplemented with 10% fetal 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 1 mm^3 pieces approximately. Cartilage pieces were covered by the solution of 6 mg/mL of collagenase I (Merck) 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 DMEM/F12 medium containing 10% FBS and 1% penicillin/streptomycin mixture. The cell suspension was filtrated through a 70 μm nylon membrane and centrifuged at 150 g force for 5 min. Then, the supernatant was discarded, and the 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) at the density of $5 \times 10^3/cm^2$. The cells were incubated at 37 °C in humidify atmosphere with 5% CO_2 for 5 days. After this period, the medium was exchanged, and the cells were ready for further experiments.

3.4. Cell Viability Determination

To determine cell viability, a 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 the cells were prepared, they were treated by the tested 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 the tested compounds to inhibit the transcription factor NF-κB, one of the key pro-inflammatory intracellular regulators, 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) and was dissolved in serum-free RPMI 1640 medium (1 g/mL) after 1 h pre-treatment with the tested compounds dissolved in DMSO in the concentration of 10 μM.

Author Contributions: Conceptualization, J.J.; methodology, J.H. and J.J.; investigation, J.H., P.B. and J.J.; writing, J.H., P.B. and J.J.; funding acquisition, J.H. and J.J. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the Slovak Research and Development Agency (APVV-17-0373). The work of J.H. was supported by the Ministry of Education, Youth, and Sports of the Czech Republic under the project "FIT" CZ.02.1.01/0.0/0.0/15_003/0000495, and Czech Ministry of Agriculture grant no. RO0518.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: The authors thank Cupalova for her help with a cell viability assay and to Novobilsky for his assistance with cartilage tissue preparation.

Conflicts of Interest: The authors declare no conflict of interest.

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