universität wien

DIPLOMARBEIT / DIPLOMA THESIS

Titel der Diplomarbeit / Title of the Diploma Thesis

„Biological activity of hydrogen sulfide-releasing compounds in osteoarthritic chondrocytes and synoviocytes“

verfasst von / submitted by

Natalia Udovicic

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Magistra der Pharmazie (Mag.pharm.)

Wien, 2019

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 449
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Diplomstudium Pharmazie
Betreut von / Supervisor:	O. Univ. Prof. Mag. Dr. Helmut Viernstein
Mitbetreut von / Co-Supervisor:	Assoc.-Prof. Priv.Doz. Mag. Dr. Stefan Tögel

Danksagungen

An dieser Stelle möchte ich mich bei all jenen bedanken, die mich während der Anfertigung dieser Diplomarbeit unterstützt und motiviert haben.

An erster Stelle möchte ich mich ganz herzlich bei O. Univ. Prof. Mag. Dr. Helmut Viernstein bedanken, der es mir ermöglicht hat meine Diplomarbeit im Department für Pharmazeutische Technologie und Biopharmazie, Universität Wien, absolvieren zu können.

Mein herzlicher Dank gilt Assoc.Prof. Priv.Doz. Mag. Dr. Stefan Tögel, für die Gelegenheit meine Diplomarbeit im Karl Chiari Lab for Orthopaedic Biology durchführen zu können. Ich danke dir Stefan für deine wunderbare Betreuung und das äußerst interessante Thema meiner Diplomarbeit! Danke, für deine Mühe, deine Geduld und, dass du während der gesamten Zeit immer für mich da warst. Ich hätte mir einfach keinen besseren Betreuer wünschen können!

Ebenso gilt mein Dank auch dir, Kathi! Ich danke dir, dass du immer ein offenes Ohr für mich hattest und immer sehr bemüht warst, wenn ich deine Hilfe benötigt habe.

Vielen herzlichen Dank auch an das restliche Team des Labors! Vor allem danke ich dir Meli, dass du so viel Geduld und Freude bei der praktischen Einschulung mit mir hattest. Vielen lieben Dank auch euch Sonja, Ruth und Melanie, dass ihr mir die Zeit hier so angenehm und lustig gestaltet habt. Ohne euch wäre die Zeit im AKH halb so lustig gewesen!

Vielen Dank auch dir, Danyal! Ohne dich wäre das Studium wahrscheinlich doppelt so schwer für mich gewesen! Ich danke dir für deine Unterstützung, deine Motivation, deine Hilfe und deine Liebe während der letzten Jahre.

Herzlichen Dank auch an meine Mädels Raphi, Rebbi, Lin, Lisa, Magda, Nati und Johanna! Egal wie oft ich mich die ganzen Jahre bei euch über mein Studium beschwert hab, habt ihr mir immer gut zugesprochen und immer an mich geglaubt und mich nie aufgegeben.

Ebenfalls möchte ich mich auch bei meiner lieben Schwester bedanken! Ich danke dir Alexandra, dass du mich auch in schlechten Zeiten immer aufmuntern und zum Lachen bringen konntest.

Abschließend möchte ich mich herzlichst bei meinen Eltern bedanken, die mir das Studium durch ihre Unterstützung ermöglicht haben und stets an mich geglaubt haben. Ich danke euch für alles und werde euch für eure Liebe immer dankbar sein!

Table of Contents

Abstract

Osteoarthritis (OA) is the most common type of arthritis affecting millions of people worldwide. OA is characterized by a progressive joint degeneration leading to symptoms such as joint pain and stiffness. Since there is no cure, the interest in the development of potential pharmaceutical treatments is increasing. Hydrogen sulfide (H_2S) is a regulator in inflammatory processes and might offer therapeutic value in the treatment of OA.

Therefore, the aim of this study was to evaluate the effects of two H_2S-releasing compounds DP* and NaHS in OA chondrocytes and OA synoviocytes. Experiments with a H_2S-specific fluorescence probe showed that NaHS released H_2S immediately in solution, whereas DP* could be activated only in presence of glutathione (GSH) or cysteine. Further, viability assays (MTT) were performed to assess the highest concentration of DP* and NaHS tolerated OA chondrocytes and OA synoviocytes. After this, we analyzed gene expression with RT-qPCR and focused on the effects of DP* and NaHS on IL-1β-induced IL6 and MMP13 mRNA levels. Our results showed that DP* suppressed the IL-1β-mediated up-regulation of OA markers, whereas NaHS and the precursor D-penicillamine could not show any similar effects. Moreover, in comparison to DP*, the combination of DP* and GSH revealed an alleviated down-regulation of IL-1β-induced IL6 and MMP13 mRNA levels. In addition, In-Cell Western (ICW) experiments were performed to investigate the involvement of DP* and NaHS in NF-κB and CREB signaling.

In summary, the results of the current study indicate that the novel H_2S-releasing agent DP* down-regulates inflammatory and degenerative mediators in OA chondrocytes and OA synoviocytes. Therefore, DP* might represent a promising H_2S-relesing compound in pharmaceutical applications.

Zusammenfassung

Osteoarthrose (OA) ist die häufigste Art der Arthrose, von der Millionen von Menschen weltweit betroffen sind. OA zeichnet sich durch eine progressive Gelenksdegeneration aus, die zu Symptomen wie Gelenkschmerzen und –versteifungen führt. Da es derzeit keine Heilung gibt, steigt das Interesse an der Entwicklung potenzieller medikamentöser Behandlungen. Schwefelwasserstoff (H_2S) ist bekannt für die Regulation von verschiedenen Entzündungsprozessen und könnte daher eine therapeutische Möglichkeit bei der Behandlung von OA darstellen.

Ziel dieser Studie war es daher, die Auswirkungen der beiden H_2S-freisetzenden Verbindungen DP* (Derivat von D-Penicillamin) und NaHS in OA Chondrozyten und OA Synoviozyten zu untersuchen. Experimente mit einem H_2S-spezifischen Fluoreszenzfarbstoff haben gezeigt, dass das NaHS den Schwefelwasserstoff sofort in Lösung freisetzen konnte, während DP* nur in Gegenwart von Glutathion (GSH) oder Cystein aktiviert werden konnte. Weiters wurden Zellviabilitättests (MTT) durchgeführt, um die höchste tolerable Konzentration von DP* und NaHS in den Zellen zu ermitteln. Danach analysierten wir die Genexpression mittels RT-qPCR und fokussierten uns auf die Auswirkungen von DP* und NaHS auf IL-1β-induzierte IL6 und MMP13 mRNA Levels. Unsere Ergebnisse zeigten, dass DP* die IL-1β-vermittelte Hochregulierung von OA-Markern unterdrücken konnte, wohingegen NaHS und die Vorläufersubstanz D-Penicillamin keine ähnlichen Wirkungen zeigen konnten. Im Vergleich zu DP* zeigte die Kombination von DP* mit GSH eine abgeschwächte Abnahme der IL-1β-induzierten IL6 und MMP13 mRNA Levels. Zusätzlich wurden In-Cell Western (ICW) Experimente durchgeführt, um einen möglichen Effekt von DP* und NaHS auf den NF-κB und den CREB-Signalweg zu untersuchen.

Zusammenfassend zeigen die Ergebnisse der aktuellen Studie, dass die neue H_2S-freisetzende Substanz DP* die Genexpression von entzündlichen und degenerativen Mediatoren in OA Chondrozyten und OA Synoviozyten vermindern kann. Aus diesem Grund könnte DP* eine neue vielversprechende H_2S-freisetztende Verbindung in klinischer Anwendung darstellen.

1. Introduction

1.1. The human knee joint

1.1.1. Anatomy

The human knee joint is the largest and most complex joint in the human body. It is also one of the strongest and weight bearing joints enduring a large amount of force most of the time. The knee joint connects the thigh bone (the femur), shin bone (the tibia) and kneecap (the patella), and keeps them in place. There is a fourth bone called calf bone (the fibula), which is not part of the knee joint, yet acts as an attachment donor for the muscles (quadriceps and hamstrings), which keep the knee stable (Figure 1). In fact, there are two single joints forming the knee: the tibiofemoral joint connects the tibia to the femur and the patellofemoral joint joins the femur to the patella. The ends of these bones are covered with articular cartilage for a painless and smooth movement. The two fibrocartilaginous menisci located between the tibia and femur act as shock absorbers and spread the weight of the body over a larger area. The medial meniscus is on the inner side of the knee and also the larger one. The lateral meniscus is on the outer side of the knee. Both are made of fibrocartilaginous tissue, which is the reason for its smooth and elastic surface. However, sharp and rapid motions can damage the meniscus and lead to a meniscal tear. The knee joint is surrounded by a thick, protective and fibrous membrane which is known as the joint capsule. Located at the inner surface of the capsule is the synovial tissue, which secretes the synovial fluid. This viscous liquid consists of hyaluronic acid, lubricin, collagenases, proteinases and prostaglandins. It fills the whole capsule and is responsible for the lubrication and nutrition of the articular cartilage. Moreover, in and around the knee some fluid-filled sacs, also called bursae, help to cushion the joint. The knee joint is stabilized by four different types of ligaments (the major ligaments are shown in Figure 1). Ligaments are though, strong robes made of a fibrous tissue connecting bones to other bones. On the one hand, they allow a wide range of movement while keeping the structure of the joints together. On the other hand, they also prevent movement in certain directions, like bending the knee backwards. However, not only ligaments are important for the stability of a joint, but also tendons, which connect the bones with the muscles.

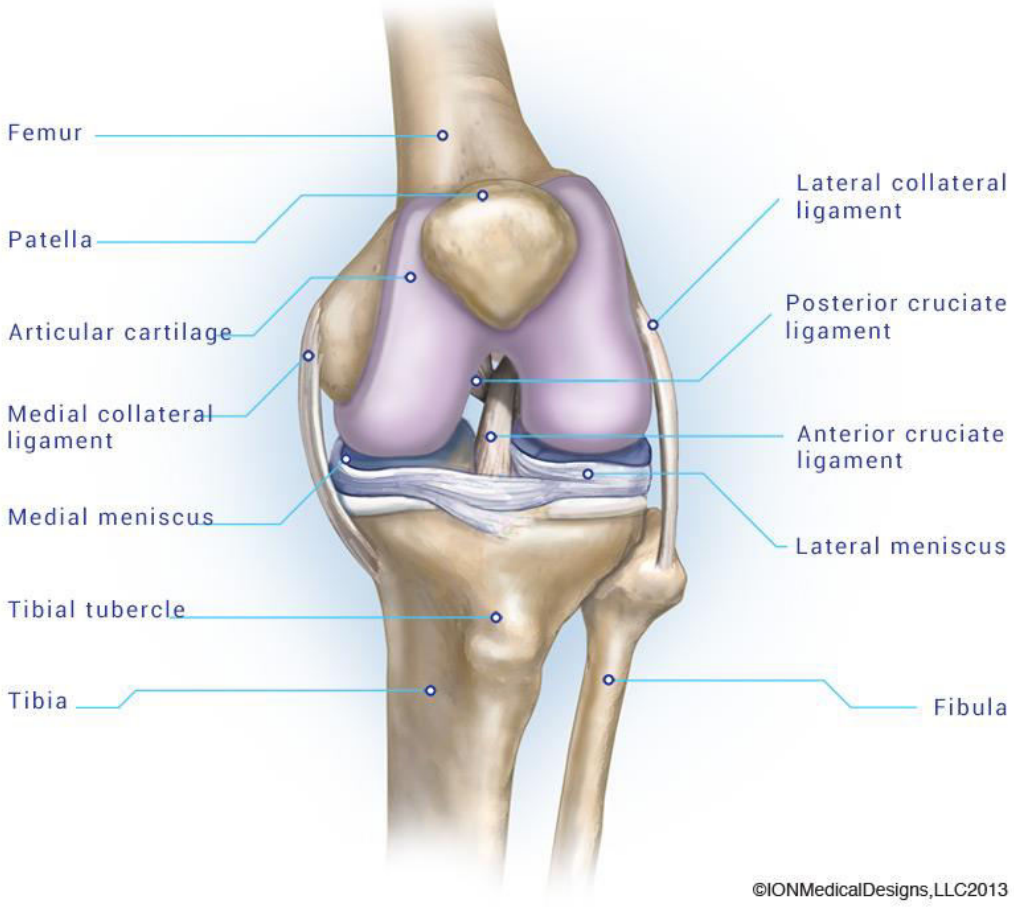


Figure 1: Human knee joint anatomy (Image taken from http://josephbermanmd.com/anatomy-of-the-knee/articular-cartilage/, 18.02.2019)

1.1.2. Articular cartilage

The main function of articular cartilage is to ensure a smooth, friction-free moving surface for synovial joints. In contrast to bone, cartilage contains no blood vessels or nerves, limiting the supply with nutrients to diffusion and osmosis. The cartilage cells are called chondrocytes and represent the only living component of the cartilage. They are metabolically active cells that maintain and secrete a large amount of extracellular matrix (ECM). Chondrocytes originate from mesenchymal stem cells and compose about 1-5% of the total volume of articular cartilage. The metabolic activities of chondrocytes are affected by many factors such as the pro-inflammatory cytokines and growth factors that have anabolic and catabolic effects. These factors are responsible for the degradation but also for the synthesis of matrix macromolecules.

Extracellular matrix (ECM) is composed of proteoglycans, collagen and glycoproteins. Aggrecan is one of the major and largest proteoglycans in articular cartilage. It is composed of keratin sulfate glycosaminoglycan (GAG) and chondroitin sulfate that are linked to a large protein core. This molecule has the ability to interact with hyaluronic acid via a link protein and to stabilize proteoglycan aggregates. These aggregates are able to attract and maintain water in the ECM. Water makes up to 80% of the weight of articular cartilage and is important for compression resistance.

The cartilage can be divided in four different zones: the superficial zone, the middle zone, the deep zone and the calcified zone (Figure 2).

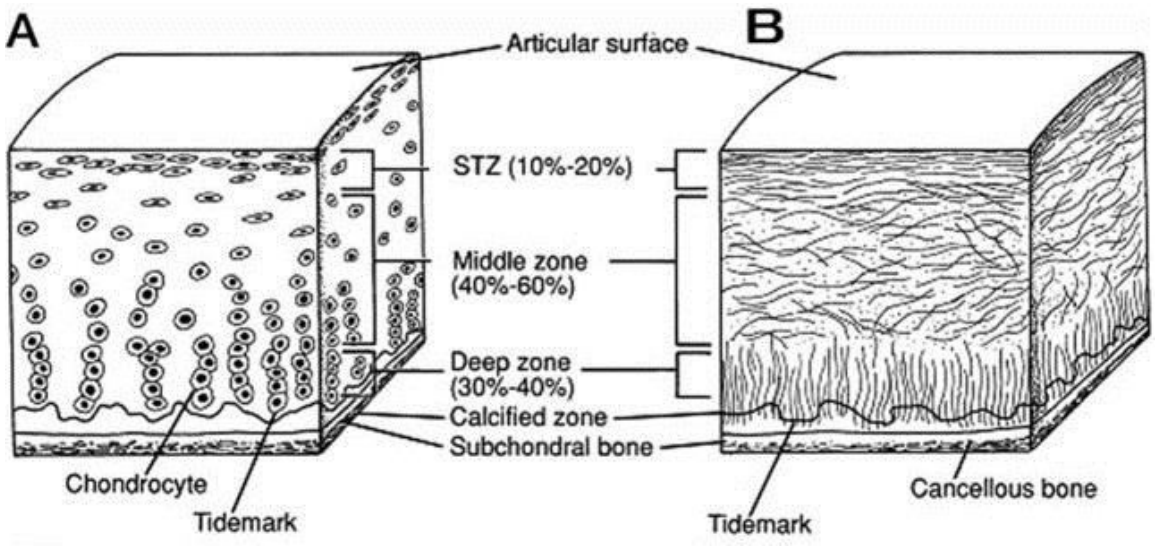


Figure 2: Cross section of an articular cartilage (Image taken from Sophia Fox et al. 2009)

The superficial zone makes up to 10% of the cartilage and is thus the thinnest one. Here, the collagen fibers are oriented parallel to the articular surface and mainly responsible for protection of the deeper layers. The middle zone is the largest one and located directly under the superficial zone. This zone has thicker collagen fibers and a high proteoglycan and water content. The deep zone, which is located one layer beneath, contains large collagen fibers vertical to the articular surface and many chondrocytes. Together, the middle and deep zone, act as a compression-resisting core. The tide mark is a basophilic line that separates the deep zone from the calcified cartilage. The calcified zone establishes a connection between the cartilage and the subchondral bone.

1.2. Osteoarthritis

Osteoarthritis (OA) is a degenerative joint disease and the most common form of arthritis, affecting approximately 10% of men and 18% of women over 60 years all over the world (Glyn-Jones et al. 2015). This long-term chronic disease, characterized by a multifactorial process, leads to an alteration of articular cartilage (Figure 3). Mainly the protecting cartilage gets thinner over time and the bones of the joint begin to press against each other causing progressive loss of function and pain. Therefore, OA is often associated with mobility disability and decreased quality of life.

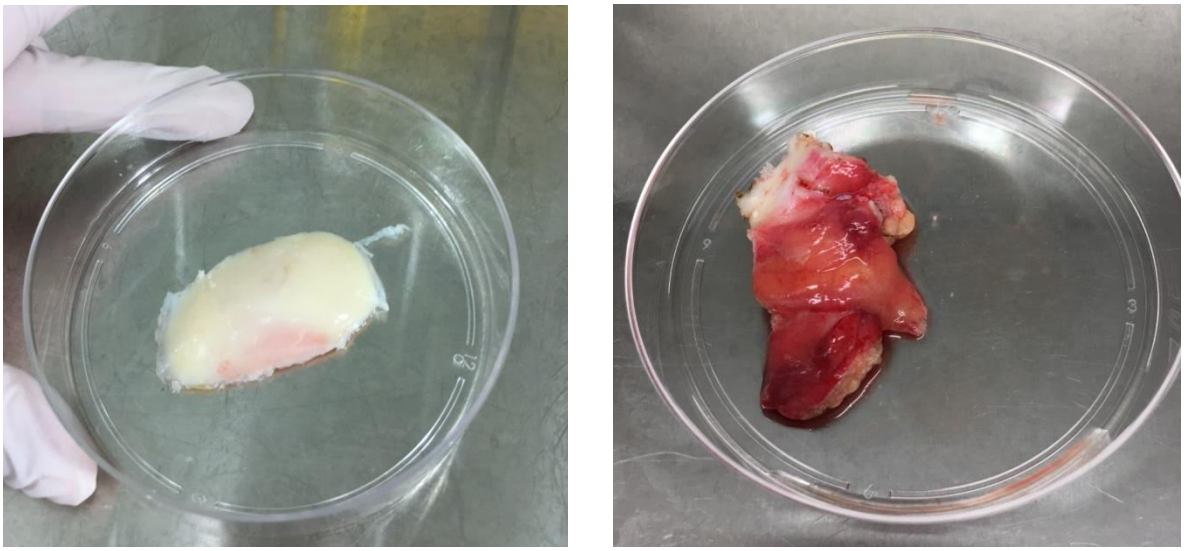


Figure 3: The left image shows a femoral condyle and the right image shows synovial tissue of a human knee joint. Both were received from an OA patient that underwent arthroplasty.

OA can affect any joints, but it occurs most commonly in hands, knees, hips and spines. This complex disease affects the whole joint, including cartilage, synovium, subchondral bone, menisci, ligaments, periarticular muscle and capsule. These patients suffer from pain, transient stiffness, reduced movement, joint crepitus and cartilage swelling. An advanced osteoarthritis can be detected with plain radiographs and is characterized by joint space narrowing, osteophytes and also alternations in the subchondral bone (Hunter and Felson 2006).

The main risk factors are increasing age, obesity, female gender, inactive lifestyle, joint injuries, incorrect posture or occupation (for example joint-intensive sports can lead to

OA). However, a genetic factor may also play an important role in the onset of osteoarthritis (Sharma et al. 2013).

1.2.1. Pathogenesis

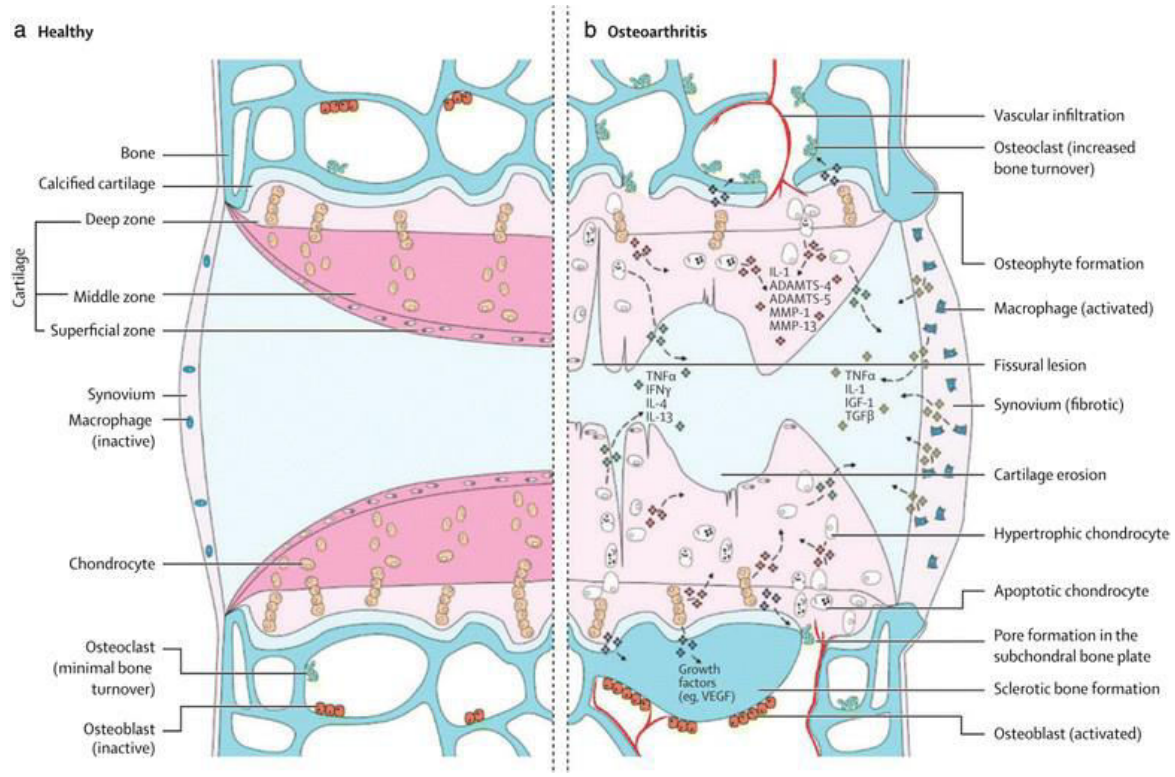


Figure 4: Comparison of the anatomy and structural changes between a healthy and an osteoarthritic knee joint. (Image taken from Glyn-Jones et al., 2015)

For a long period of time, osteoarthritis has been described as a purely mechanical irreversible degeneration of the cartilage. Nowadays, it is known that OA is a very complex process, where an alternation in the joint environment leads to an activation of inflammatory cytokines, chemokines and catabolic genes (Figure 4). Inflammation plays an important role in the genesis of the disease and is the main reason for progression of cartilage loss and also for signs and symptoms of OA (Goldring and Otero 2011).

Synovitis is therefore often a characteristic symptom even in an early stage of OA, which leads to synovial hypertrophy and production of inflammatory mediators and degradative enzymes in an established OA (Mathiessen and Conaghan 2017). However, not only

synovium but also activated chondrocytes are able to release cytokines, including mainly interleukin 1β (IL-1β), interleukin 6 (IL-6) and tumor necrosis factor-α (TNF-α), as well as several matrix-degrading enzymes, like metalloproteinases (MMPs) or- disintegrin and metalloproteinase with thrombospondin-1 domains (ADAMTS) (Goldring and Otero 2011). In healthy cartilage some of them have positive effects in terms of remodeling, which is important to maintain tissue homeostasis. On the other hand, in osteoarthritic cartilage some of them are involved in the pathophysiological processes, such as metalloproteinases 1, 3, 13 and ADAMTS 4 and 5 (Glyn-Jones et al. 2015).

1.2.2. Non-pharmacological therapy

Although osteoarthritis is a disease with no cure, there are many options to reduce the progress and to relieve the symptoms and pain of the patients. At the beginning of the pain management, a nonpharmacological therapy should be considered (Figure 5).

Firstly, it is important to educate patients about the risk factors just like obesity, because extra body weight increases pressure mainly on weight-bearing joints, such as hips and knees. Therefore, overweight patients should be encouraged to lose weight through a healthy and active lifestyle (Hunter and Felson 2006). Physical exercise not only reduces weight, but also improves function and relieves pain of OA patients. A combination of aerobic, strengthening and functional exercise with direct therapist contact is recommended to patients with OA. Strengthening the muscles can support and protect affected joints mainly of patients with knee OA. However, any kind of exercise shows beneficial effects on pain and movement as long as it is discussed with a physiotherapist and/or performed correctly (Page et al. 2011).

Another non-pharmacological treatment, which is recommended to patients with knee OA, is knee taping. It is believed, that taping can reduce pain by improving the patellar alignment and unloading painful soft tissues. However, patients should be enlightened by the doctors or therapists about the correct application, because wrong usage can cause skin damage, mainly in older patients.

Braces or shoe inserts can offer more stability and minimize knee load mainly for patients with unicompartmental OA. Braces can support the joint and relieve pain by taking pressure off a painfully affected area of a joint (Page et al. 2011).

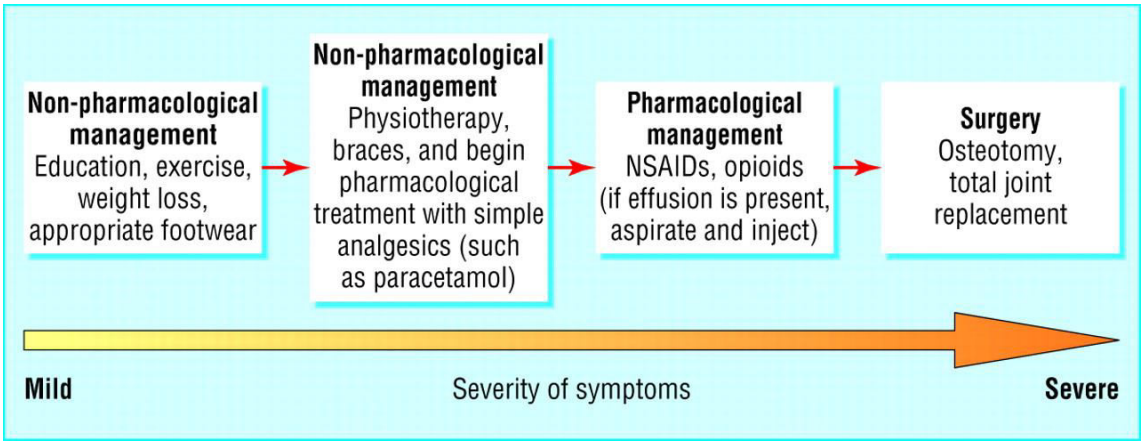


Figure 5: Stepwise pain management of OA (Image taken from Hunter and Felson, 2006)

Moreover, thermotherapy is also a non-pharmacological, inexpensive and helpful way to reduce pain, which can be done with packs, wax, towels and more. Heat can relax muscles and relieve joint stiffness whereas cold can reduce inflammation and swelling. An alternating heat and cold therapy can also improve the symptoms of osteoarthritis (Brosseau et al. 2003)

1.2.3. Pharmacological therapy

As joint degeneration gets worse, the pain gets much more intense and often a combination of a nonpharmacological and pharmacological therapy may be needed to control the pain. The choice of the pharmaceutical painkiller depends on the pain intensity and on patient´s health condition.

For patients with mild to moderate pain, paracetamol is the first-line therapy to begin with. Paracetamol, also known as acetaminophen, is an oral analgesic, which can be bought without prescription in pharmacies. As long as the recommended dose of up to 4g a day is not exceeded, it is an effective drug with an excellent safety profile (Hunter and Felson 2006). However, overdosing with paracetamol or combined use of paracetamol with alcohol can cause liver damage. Non-steroidal anti-inflammatory drugs (NSAIDs) are mainly prescribed for patients with moderate to severe pain, because of a greater efficacy and an additional anti-inflammatory effect (Zhang et al. 2016). However, NSAIDs such as ibuprofen, naproxen or diclofenac are cyclooxygenase 1 and 2 (COX-1/2) inhibitors, which not only relieve pain and decrease inflammation, but also lead to renal and gastrointestinal

complications due to the prostaglandin inhibition. In worst case, patients, who routinely use NSAIDs, can suffer from ulcers or gastrointestinal bleedings. For this reason, it is recommended for these patients to take NSAIDs in combination with a gastroprotective agent, such as proton pump inhibitors (PPIs), to help prevent an ulcer. Furthermore, COX-2 selective inhibitors, called coxibs, are also an option for patients with severe pain issues. Coxibs, such as rofecoxib or etoricoxib, has been developed with the intention to reduce the disadvantages of non-selective inhibitors and lower the renal and gastrointestinal complications. However, later it turned out that they have even greater side effects and that there is no evidence of risk reduction for adverse gastrointestinal events either. In many trials, coxibs show an increased risk for serious cardiovascular diseases, such as myocardial infarction (Haq et al. 2003). Therefore, older patients or patients with cardiovascular diseases should avoid taking these drugs.

When paracetamol or NSAIDs are contraindicated or ineffective, opioid analgesics, such as tramadol, are an alternative treatment to ease pain. Due to their side effects, like constipation, nausea or dizziness and their dependency risk, opioids belong to second line therapy of OA (Zhang et al. 2016).

Moreover, some NSAIDs are available for topical application, which is a good option to reduce pain and swelling in knees and hands, but also minimize the adverse events. Topical capsaicin is an alternative for patients, who do not respond well to NSAIDs. Capsaicin blocks the nerves that are responsible for pain messages in the applied area and relieves pain in knees or hands (Haq et al. 2003).

Beside the oral and topical treatments, intra-articular injections of corticosteroids or hyaluronic acid have also a good efficacy in OA. The amount of hyaluronic acid, which is a component of synovial fluid and cartilage, is decreased in OA. Studies have shown, that supplementation of hyaluronic acid with intra-articular injections can reduce pain after some weeks (Haq et al. 2003).

1.2.4. Surgery

A total joint replacement surgery, also known as arthroplasty, is the last solution for pain relief for patients with a severely damaged joint (Hunter and Felson 2006). It is a surgical replacement of damaged joint surfaces with prosthesis or an artificial joint, which restores the function of a joint. The most frequently performed surgeries are hip and knee replacements (Figure 6), but it is also possible to replace a joint in the shoulder, ankle, wrist or elbow. Arthroplasty usually increases the quality of patient´s life due to functional improvement and pain relief (Katz et al. 2010).

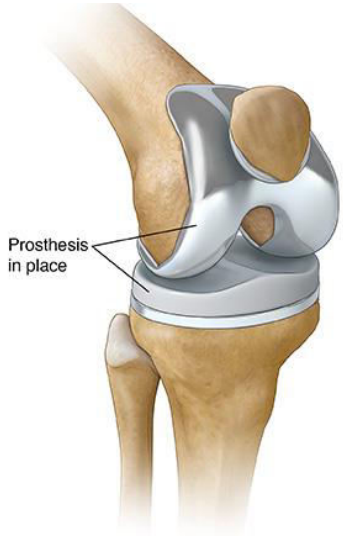


Figure 6: Total knee joint replacement (Image taken from https://www.saintlukeskc.org/condition/total-knee-replacement, 18.02.2019)

1.3. Hydrogen sulfide (H_2S)

1.3.1. Properties

Hydrogen sulfide (H_2S), a flammable, colorless, high toxic gas, is rapidly recognized even in low concentrations by the smell of rotten eggs. It is known as a toxic pollutant but also as a gas-transmitter in mammalian body, including humans.

In low concentrations it affects mainly the respiratory system which can lead to mild symptoms like sore throat, caught, headache or shortness of breath. However, high concentration and long exposure can cause shock, coma or even death. Therefore, the severity of symptoms is dose-dependent.

As a lipophilic molecule, H_2S is able to permeate through membranes without any membrane transporters (Shen et al. 2015). It is a weak acid (pKa= 6.9), which is slightly soluble in water but very unstable in solution. At physiological pH (=7.4), it appears to 1/3 as the neutral molecule H_2S, and to 2/3 dissociated to H+ and HS- (Whiteman and Winyard 2011).

Dissociation of H_2S:

$$H_2S \leftrightarrow H^+ + HS^-$$

At this point, it isn´t identified whether the biological effects are caused directly by H_2S itself or by its dissociated form. However, it could be also possible that a combination of this two leads to the known effects of H_2S (Whiteman and Winyard 2011).

1.3.2. Biosynthesis of H_2S

The small endogenous gas-transmitter H_2S is also produced endogenously in a mammalian organism and has important physiologic functions, akin to the two transmitters nitric oxide (NO) and carbon monoxide (CO).

The biosynthesis of H_2S can occur through two pathways: an enzymatic and a non-enzymatic way. At least two key enzymes, known as cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE), can generate H_2S from L-cysteine and L-homocysteine (Kolluru et al. 2013). Both of them are pyridoxal-5'-phosphate (P5P) -dependent, which is the active form of Vitamin B6. CBS levels are predominant in brain, nervous system and liver, whereas CSE is active in aorta and other peripheral tissues. Lately, 3-mercaptopyruvate sulfur transferase (3-MST) has been discovered, which can also synthesize H_2S in combination with cysteine aminotransferase (CAT) and α-ketoglutarate (α-KG) as a co-factor, but has a lower physiological relevance in an organism than the other two enzymes. Moreover, 3-MST generates H_2S in vasculature and brain and can only use L-cysteine as its substrate for the H_2S biosynthesis (Figure 7). In addition, CBS and CSE are mainly located in the cytosol, whereas 3-MST can be found in cytosol but also in mitochondria. However, all these enzymes work together on the H_2S homeostasis in a mammalian system. A non-enzymatic production of H_2S through glutathione, glucose, elemental sulfur and polysulfides is also known, but less understood in contrast to the enzymatic synthesis (Kolluru et al. 2013).

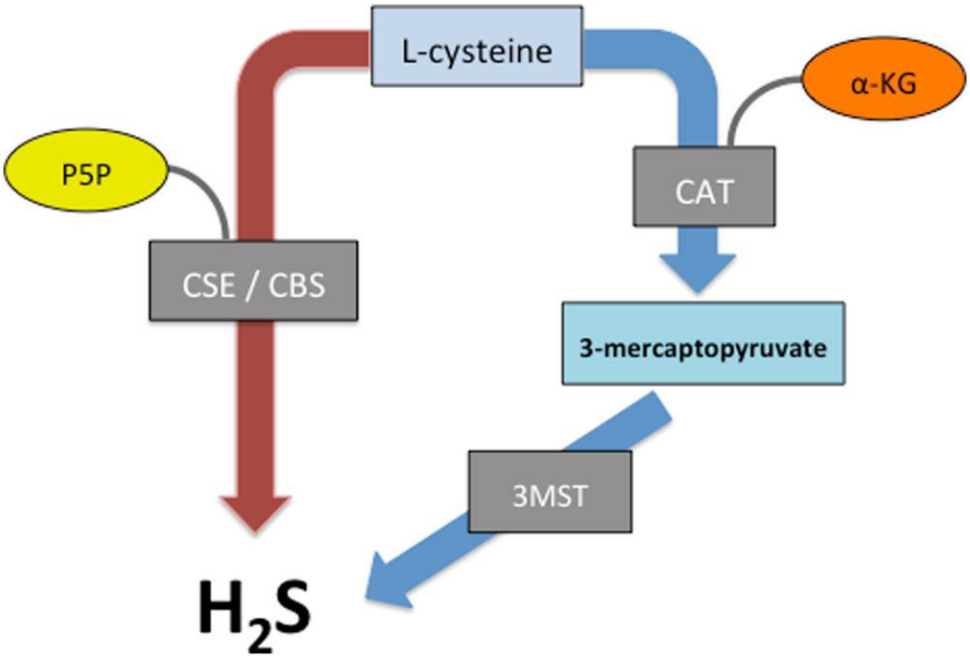


Figure 7: H_2S synthesis by CBS, CSE and 3-MST (Image taken from Flannigan et al., 2013)

1.3.3. H_2S-signaling

Sulfhydration is a physiologic posttranslational modification of proteins and the main molecular mechanism of H_2S signaling inside the cells. It is very similar to nitrosylation, since both of them are reversible by the thioredoxin system and perform on reactive cysteine residues. However, sulfhydration occurs more often than nitrosylation and they also differ from each other in terms of protein activity modulation. In short, the two modifications of proteins result in opposite effects, although they occur on the same residues. During sulfhydration, H_2S converts free thiols, with a low p*K*a, of specific cysteines (-SH) to hydropersulfide (–SSH) through addition of sulfur atoms (Paul and Snyder 2012). In comparison, hydropersulfide has enhanced chemical reactivity than thiols, due to stronger acidity, whereas nitrosylation of proteins usually inhibits their activity (Gadalla and Snyder 2010). This phenomenon was first observed in the regulation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which is the key regulatory enzyme of glycolysis. Here, nitrosylation of the GAPDH on Cys150 residues negates its activity. In contrast, sulfhydration of GAPDH markedly increases its activity (Paul and Snyder 2012).

1.3.4. Physiological and pathological effects of H_2S

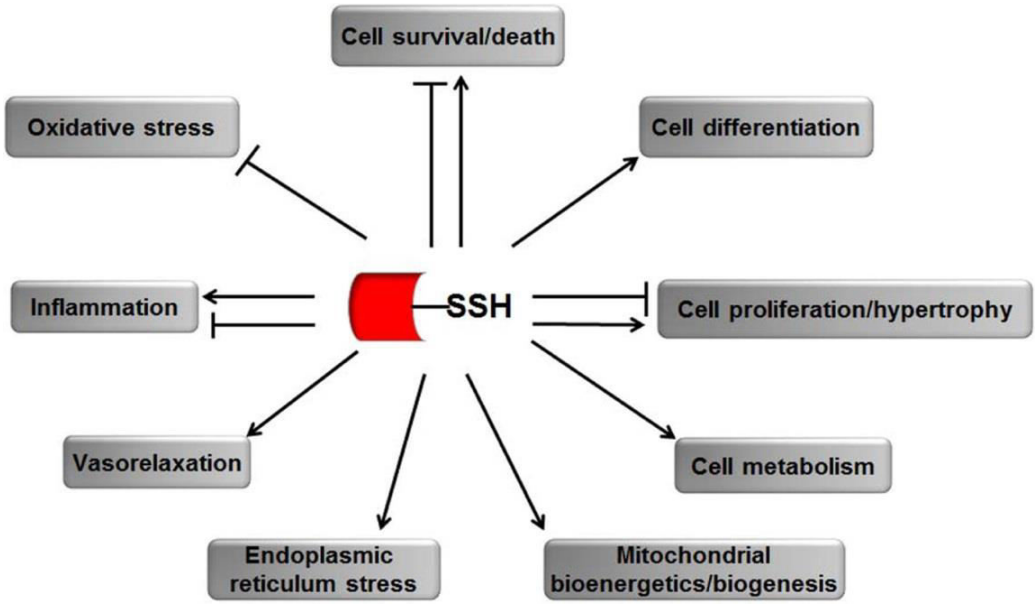


Figure 8: Effects of H_2S: → means stimulation, ⊣ means inhibition (Image taken from Zhang et al., 2017)

Hydrogen sulfide regulates a lot of physiological and pathophysiological processes in a mammalian body (Figure 8). However, its roles in cardiovascular system and inflammatory processes are probably the most important ones (Zhang et al. 2017).

The first known beneficial biological effect of H_2S is that it can reduce pain and inflammation. Regarding pain relief, natural sulfur springs have been popular remedies for patients with inflammatory diseases. Due to soaking in sulfur water, the human body is able to absorb sulfur transdermally and alleviate the pain and swelling of OA, rheumatoid arthritis and psoriatic arthritis. For a long period of time the molecular mechanisms were unknown, however in the last few decades much research has gone into the role of H_2S in inflammatory processes.

It was observed that H_2S synthesis was up-regulated due to an increased tissue CSE and CBS expression levels in several gastrointestinal tissue injuries, such as ulcer or colitis. When inhibiting these enzymes the healing process of ulcer was impaired, whereas the administration of H_2S-donors improved the ulcer healing. Moreover, in inflamed colonic tissues, H_2S-donors could reduce the expression of several pro-inflammatory cytokines, such as TNF-α, IL-1β or IL-8. Furthermore, H_2S-donors can also inhibit the leukocyte adherence to the vascular endothelium, which normally leads to leukocyte extravasation and edema (Gemici and Wallace 2015).

As a third member of the gas-transmitter family, H_2S induces vasodilatation as well as the other two gas-transmitters NO and CO. In contrast to NO, the H_2S-induced dilatation of blood vessels is mediated by sulfhydration of a subunit of ATP-dependent potassium channel (K_{ATP}). In smooth muscle cells this K_{ATP} channel opens after its stimulation and leads to a hyperpolarization and relaxation (Vandiver and Snyder 2012). This relaxation of blood vessels can increase blood flow, lower blood pressure and reduce hypertension as well as infarct risk.

In addition, H_2S is also an important mediator of gastrointestinal defense due to its cytoprotective actions. The administration of exogenous H_2S can protect the stomach from injuries in a multifactorial way. It is able to neutralize gastric acid, increase gastric mucosal blood flow and increases the expression of COX-2. All these effects together can lead to an acceleration of ulcer healing (Gemici and Wallace 2015).

1.3.5. H_2S-releasing agents

- **Sulfide Salts**

Hydrogen sulfide releasing agents (=H_2S-donors) are able to release H_2S under specific conditions and imitate the activities of endogenously generated hydrogen sulfide. In many studies over the years, inorganic sulfide salts like sodium hydrogen sulfide (NaHS) and sodium sulfide (Na_2S) have been examined as H_2S donors (Figure 9) (Zhao et al. 2014). In this case, the compounds release H_2S immediately and completely in solution due to simple hydrolysis and after the release they do not form any by-products, so that they fall into the category of "safe" donors. However, the volatility of sulfide salts in solutions causes an uncontrollable loss of H_2S under laboratory conditions, which reduces the residence time of H_2S in tissues and experiments. In addition, the undesirable odor of sulfide salts can affect the compliance of patients and also the pharmaceutical development (Zheng et al. 2018).

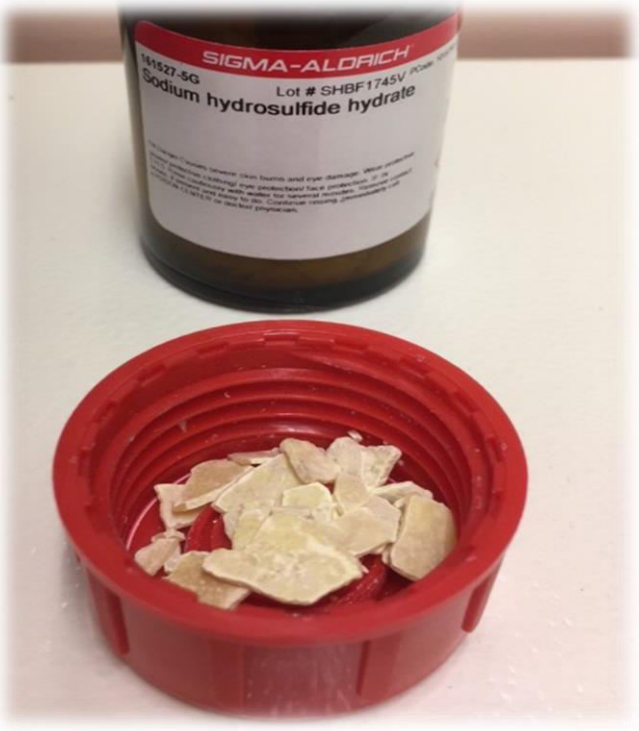


Figure 9: Flakes of NaHS

- **Prodrugs**

Because of the problematic issues associated with sulfide salts, a number of new H_2S-releasing compounds or prodrugs with unique features were introduced in the last few years. Figure 10 summarizes the different types of H_2S-prodrugs (Zheng et al. 2018).

The ideal H_2S prodrug should have characteristics such as:

→ good water solubility
→ good stability
→ odorless
→ triggered release
→ harmless “side products”

The most challenging issue is probably the development of a prodrug that generates H_2S at a slow and steady state so that it can mimic endogenous H_2S release. Another difficulty is also to achieve a localized release of H_2S.

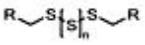
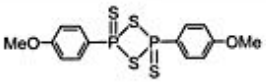
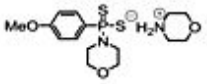
H_2S prodrugs/donors	Chemical structures	Proposed H_2S release mechanism	Representative bioactivities	a: good water solubility; b: triggered release; c: amenable for structure modification; d: tunable release rates; e: well-defined control compounds f: good stability; g: odorless Y= Yes, N= No, M= more studies needed, V= Variation in properties based on the different donors						
				a	b	c	d	e	f	g
Hydrogen sulfide gas	H_2S	Direct H_2S resource	Induction of suspended animation-like state, Protection against lethal hypoxia, Increased glucose uptake, Protection against lethal hemorrhage, Reversible depression of cardiovascular function	Y						
Sulfide salts	NaHS, Na_2S	Hydrolysis	Increased glucose uptake, Protection against gastropathy, Protection against lethal hemorrhage, Protection against MI/R injury, Inhibition of HIF-1 α expression, Anti-thrombotic	Y					M	
		Hydrolysis	Cytoprotection against H_2O_2-induced damage	Y					M	M
Natural sulfide releasing compounds		Thiol activation	Cardioprotection, Anti-inflammatory, Neuroprotection, Pro-angiogenic	V	Y	Y				
SG-1002	 α-sulfur	No reported	Cardioprotection	Y					M	
Lawesson's Reagent		Hydrolysis	Anti-inflammation (colitis), Protection against gastric damages, Ion channel regulation	N		N			M	
GYY4137		Hydrolysis	Vasorelaxation, Anti-cancer, Anti-inflammatory, Protective effect against H_2O_2 -nduced oxidative injury	Y					M	
Phosphorodithioates		Hydrolysis	Prevention against oxidative damages	Y		Y			M	Y
Thioamide		Thiol activation	Vasodilation	V	M	Y			M	V
Isothiocyanate		Thiol activation	Vasodilation		Y	Y				Y

Figure 10: Summary of current H_2S donors on the market (Image taken from Zheng et al., 2018)

- **DP***

DP* is a novel H_2S prodrug, which was discovered by the French chemist Erwan Galardon. DP* is an ammonium salt synthesized by a chemical reaction of the substances L-penicillamin and methoxycarbonylsulfenyl chloride. The compound is soluble in aqueous buffers but it also shows moderate stability in solutions due to limited degradation. In contrast to sulfide salts, it is suggested that DP* gets activated by the presence of free thiols such as cysteine or glutathione, which should assure a more controlled release of H_2S than inorganic sulfide salts (Artaud and Galardon 2014).

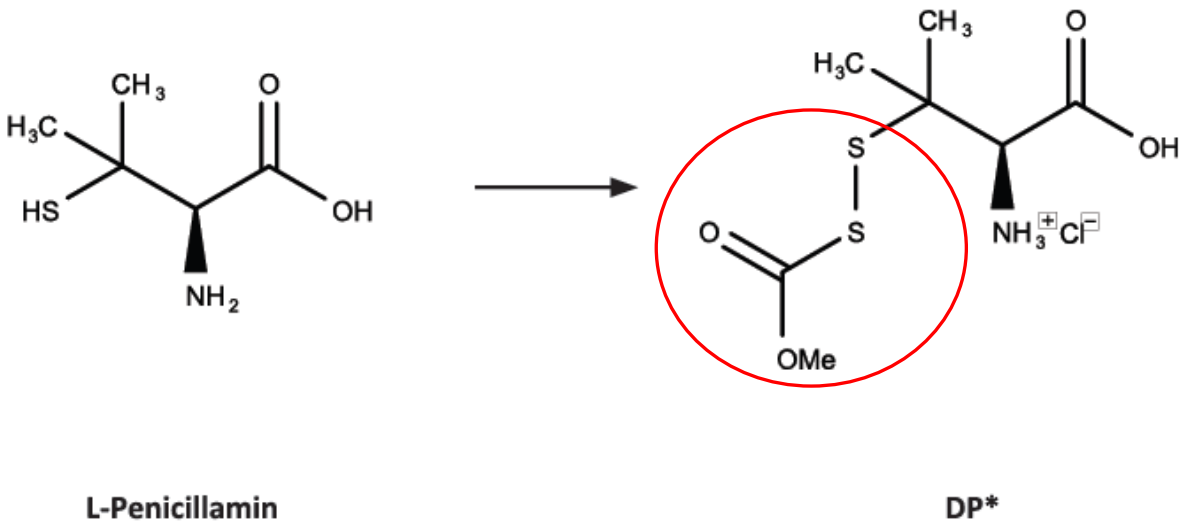


Figure 11: Structure of the precursor substance L-penicillamine (left side) and DP*(right side)

1.3.6. H_2S-based therapeutics

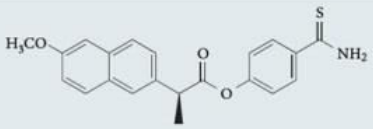
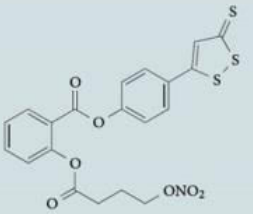
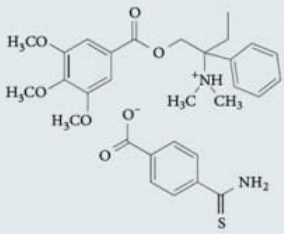
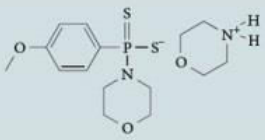
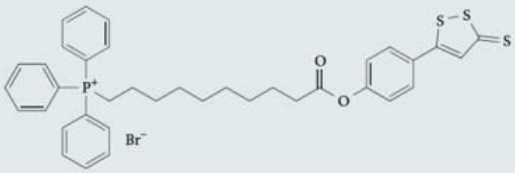
Many companies focused on the development of new H_2S-based therapeutics due to the known benefits of H_2S in human organism (Figure 12).

In recent years, scientists have mainly focused on the development of H_2S-releasing NSAIDs, based on the knowledge that these drugs demonstrated similar effectiveness to conventional NSAIDs. In vivo they have shown an inhibition of COX-1 and COX-2, which was very comparable to the effects of parent NSAIDs. However, the main beneficial effect of H_2S-releasing NSAIDs over conventional NSAIDs is the improved gastrointestinal tolerability. H_2S-NSAIDs do not affect the gastric mucosa as much as the common NSAIDs and are therefore much safer to use for a long-term medication. The main indications of these H_2S-releasing NSAIDs are diseases such as osteoarthritis, where they can reduce gastrointestinal bleedings and ulceration of the patients (Gemici and Wallace 2015).

In addition, the City University of New York developed H_2S-based NSAIDs with the main focus on chemoprevention of cancer. Beside their decreased gastrointestinal toxicity, they could also show a greater efficacy than their precursors (Wallace and Wang 2015).

Another important target of H_2S-based drug development is cardiovascular diseases. The compounds SG-1002 and AP39 have shown promising results in reduction of oxidative stress and inflammation. These H_2S-donors are able to release H_2S inside of mitochondria, where they can downregulate the antioxidant response pathway. Therefore, these therapeutics could be useful future treatments for myocardial infarction, heart failure or hypertension (Wallace and Wang 2015).

Furthermore, GIC-1001 is another H_2S-based drug in phase II clinical trials, with the aim to replace sedation during endoscopy due to its visceral analgesic effects. It is a salt made of trimebutine and thiobenzamide - as its precursor trimebutine - , its indication may be also extended for the treatment of irritable bowel syndrome (Gemici and Wallace 2015).

Institution (location)	Structure	Clinical indications	Lead drug	Comment	Stage of development
Antibe Therapeutics (Toronto, Ontario, Canada)	 ATB-346	Osteoarthritis	ATB-346	Naproxen derivative	Phase I
		Acute pain	ATB-352	Ketoprofen derivative	Preclinical
		Veterinary (pain)	ATB-338	Diclofenac derivative	Preclinical
		Thrombosis	ATB-350	Aspirin derivative	Preclinical
City University of New York (New York, USA)		Cancer	NBS-1120	Aspirin derivative	Preclinical
Glcare Pharma (Montreal, Quebec, Canada)		Colonic pain	GIC-1001	Trimebutine salt; licensed from Antibe Therapeutics	Phase II for analgesia during colonoscopy*
National University of Singapore (Singapore)		Hypertension, inflammation, cancer	GYY4137	Slow-releasing H_2S donor	Unknown
Sova Pharmaceuticals (La Jolla, California, USA	No structure available	Pain, metabolic disorders	Unknown	Inhibitor of CSE activity	Unknown
SulfaGENIX (New Orleans, Louisiana, USA)		Oxidative stress	SG-1002	Polyvalent sulfur	Phase II for heart failure‡
University of Exeter (Exeter, UK)		Inflammation, oxidative stress	AP39	Mitochondrion-targeted H_2S release	Preclinical

CSE, cystathionine γ-lyase; H_2S, hydrogen sulfide. *ClinicalTrials.gov identifiers: NCT01926444 and NCT02276768. ‡ClinicalTrials.gov identifier: NCT01989208.

Figure 12: H_2S-based therapeutics in development (Image taken from Wallace and Wang, 2015)

1.4. Aims of the study

The main objective of this study was to investigate the effects of two H_2S-releasing compounds DP* and NaHS in OA chondrocytes and OA synoviocytes. In recent years, H_2S-releasing compounds received considerable attention, due to the beneficial biological effects of H_2S. Therefore, the interest in development of a potential H_2S-based therapeutic in OA is increasing. For this purpose, the aim of these experiments was to analyze the H_2S release in solution, and to study the effects of DP* and NaHS on cell viability, on mRNA levels of OA markers and on NF-κB and CREB signaling.

2. Materials and methods

2.1. Cell culture

2.1.1. Isolation of human chondrocytes/synoviocytes

Cartilage and synovial tissues were obtained from OA patients undergoing total knee joint replacement surgery with written informed consent and in accordance with the terms of the ethics committee of the Medical University of Vienna (EK-No. 1822/2017). These anatomical specimens were received from the operating theatre in sterile physiologic salt solution. After evaluation of the received tissues, useful parts were used for cell culture, whereas the leftovers and too damaged parts were given to histological examination.

At first, the cartilage was washed with phosphate buffered saline (PBS) and then resected with scalpels on petri dishes from femoral condyles, tibial plateaus and patella. The small sheets of cartilage were then cut into small pieces and added to a 50 ml Falcon tube with 300 U/ml lyophilized collagenase type II (Gibco by Life Technologies). The synovial tissue was also dissected into little pieces and added into a different Falcon tube. The cartilage and synovia were treated separately but with equal working conditions. The Falcon tubes were shaken overnight at 37°C. On the next day, the contents of the Falcons were filtered through a 40 µM nylon cell strainer to remove the undigested leftovers of the tissue. The filtrate was centrifuged at 1000 rpm for 8 min, and the cell pellet was resuspended in PBS and then centrifuged once again. Afterwards, the cell suspension was counted in a Neubauer hemocytometer (C-Chip).

2.1.2. Cell counting

Neubauer hemocytometer is a thick microscope slide with two counting-chambers where cells in a liquid sample can be counted independently. Therefore, each chamber includes a counting grid consisting of squares in different sizes (Figure 13). Only cells in the four large squares in each corner were counted to calculate the concentration of cells in the sample. For this reason, trypan blue and cell suspension were mixed together in equal volumes of 10

µl and then 10 µl were pipetted into a chamber of the C-Chip. Trypan blue colored dead cells blue making it possible to count only the living cells on the counting grid.

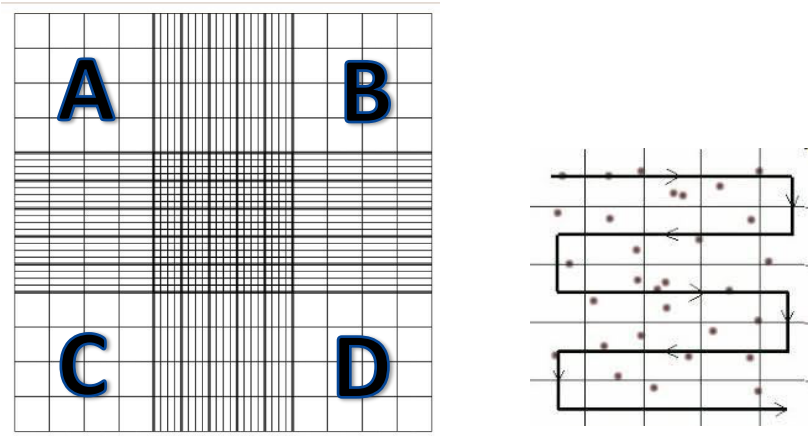


Figure 13: Counting grid (left) and counting direction of one square (right)

Cell counting formula:

Dilution with trypan blue **Predefined volume factor**

Cell concentration= (A+B+C+D): 4 * 2 * 10 * 10,000

Ø per 1mm² **Volume (ml) of the cell suspension**

2.1.3. Cell cultivation

Isolated primary OA chondrocytes and OA synoviocytes were maintained in "Full Medium" consisting of Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5g/L D-Glucose supplemented with 10% fetal bovine serum (FBS), 1% Penicillin-Streptomycin Glutamine (Pen/Strep) and Amphotericin B at 37°C. The culture medium was replaced every other day. For treatments, the OA chondrocytes were used in passage 0, whereas OA synoviocytes cultures in passage 0 still contain a number of immunocompetent cells and thus have to be passaged at least 4 times before they can be used for an experiment. At first, they were

cultured in T25 or T75 culture flasks and at a confluency of 80% they were split and placed into a new or bigger flask (T150, T225). For passaging, full medium was removed, the cells were washed with PBS and the necessary amount of trypsin/EDTA was added. The culture flask was then placed into the incubator (with an atmosphere of 5% CO_2/ 95% air at 37°C) for about 3 min. After that, 10 ml of full medium were added to the cell suspension into the culture flask to stop the enzymatic reaction. The content was transferred into a 50 ml Falcon and centrifuged at 1000 rpm for 8 min. Afterwards, the supernatant was removed and the cell pellet was resuspended in full medium and transferred into a new/bigger flask or disseminated into 96-well or 12-well plates to start an experiment.

2.1.4. Cell treatment with NaHS and DP*

Treatments of OA chondrocytes and OA synoviocytes were performed in 12-well plates for qPCR and in 96-well plates for MTT and In-Cell Western (ICW). The cultivation areas of the plates have different sizes, so the concentration of cells per cm^2 varied (Table 1). Before each treatment, except for MTT experiments, the cells were starved overnight with "starvation medium". This medium contained the same ingredients as the full medium except for FBS. As a result of serum deprivation, cells become more receptive for the substances. On the following day, the cells were treated in starvation medium with various concentrations of two exogenous sources of H_2S: NaHS and the prodrug substance DP*, which releases hydrogen sulfide (H_2S) gradually. Because of the volatility of the substances, it was important to solve the releasing agents shortly before the experiment started.

Culture plates	Medium volume	cells/well
96-well	100 µl/well	5000 - 15,000
12-well	1 ml/well	150,000

Table 1: Different cultivation plates

2.2. Cell viability assay (MTT)

To analyze cell viability in response to the treatments, we used the EASY FOR YOU (EZ4U) assay by Biomedica which is a nonradioactive cell proliferation and cytotoxicity test. At first, 5000 cells per well were cultivated in a 96-well-plate in 100 µl full medium. At a confluency of 80%, the treatment was performed in full medium for 24 h and 48 h with five concentrations of the two sulfur substances NaHS and DP*. On the next day, lyophilized substrate was dissolved in 2.5 ml activator solution and warmed -up at 37°C in the incubator. Then, full medium and the solution were mixed together at a given ratio according to the manufacturer's instructions. After removing the culture medium, each well received 110 µl of the mixture. Afterwards, the 96-well-plate was incubated for 2-3 h at 37°C to achieve a significant increase in color intensity. During the incubation, living cells convert the yellow colored tetrazolium to its red formazan derivate and then the absorbance could be measured at 450 nm using the FLUOstar optima microplate-reader.

2.3. RNA isolation

RNA-isolation is the extraction of RNA from biological samples which was accomplished with the innuPREP RNA Mini Kit 2.0. At first, 150,000 chondrocytes or synoviocytes per well were cultured in 12-well-plates. After removing the starvation medium, the cell layers were washed with PBS. Then, 350 µl lysis buffer were added into each well and resuspended by pipetting up and down to lyse the cells. The lysed material was then filtrated through Spin Filter D into a receiver tube by centrifuging for 2 min at 11,000 rpm. After discarding the Spin Filter D, the filtrate was mixed with 400 µl of 70% ethanol and transferred onto Spin Filter R placed in a new receiver tube. Then, it was centrifuged for 2 min to bind the RNA onto the membrane of the filter. The next step was washing the Spin Filter R with two different washing solutions to remove the ethanol from the membrane. After putting the Spin Filter R into a new receiver tube, 500 µl of the washing solution HS were added. After centrifugation for 1 min the Spin Filter R was placed again into a new receiver tube and washed with 700 µl of the Washing solution LS followed by a centrifugation for 1 min. At the end of the washing steps, the Spin Filter R was centrifuged for 2 min to dry the membrane. Finally, to elute the RNA from the membrane into an elution tube, 40 µl RNAse-free water were added directly

onto the membrane. After incubation for 1 min, the sample was centrifuged one last time at 11,000 rpm for 1 min. The RNA was used immediately after the isolation or stored at -80°C.

2.3.1. RNA quantification

For quantification of the total concentration and the purity (260/280 nm) of the RNA, the NanoDrop 2000 UV-vis Spectrophotometer was used at 260 nm. The samples were vortexed and pipetted on the lower pedestal and the upper arm was closed. To ensure a precise measurement, 2 µl of each sample were added. Before measuring the samples, the absorbance of RNase-free water was adjusted as blank. After each measurement, the upper and lower surfaces of NanoDrop were wiped off with a lint-free wipe to avoid contamination.

2.4. Synthesis of cDNA

Complementary DNA (cDNA) was synthesized from single stranded RNA, so it could be used for the quantitative polymerase chain reaction (qPCR). Therefore, the High Capacity cDNA Reverse Transcription Kit by Applied Biosystems was used, which contained a buffer, dNTP Mix, random primers and reverse transcriptase. At first, the samples were diluted with RNase-free water to reach the same concentration of RNA in each sample of one experiment. Afterwards, each 0.5 ml tube contained a total volume of 10 µl. In a separate step, all the necessary components for the conversion were mixed together at a given ratio (Table 2). Then, 10 µl of this Master Mix were mixed with the diluted RNA and spun down. Finally, the tubes were placed into the thermal cycler (Eppendorf Mastercycler gradient) and after choosing the right program (Table 3) the reverse transcription was started.

Component	Volume (µl)
10xRT Buffer	2.0
25xdNTP Mix (100mM)	0.8
10xRT Random Primers	2.0
MultiScribe ™ Reverse Transcriptase	1.0
Nuclease-free H_2O	4.2
Total per Reaction	10

Table 2: Master Mix components for cDNA

	Step1	Step2	Step3	Step4
Temperature	25°C	37°C	85°C	4°C
Time	10 min	120 min	5 min	persistent

Table 3: Thermal cycler program

2.5. RT-qPCR

Real-time quantitative polymerase chain reaction was used to detect and measure the quantity of DNA during each cycle of the PCR process. The detection was enabled by a double-stranded DNA binding dye (SYBR® Green) generating a fluorescent signal. The fluorescence emission increases simultaneously with the amplification of the DNA. mRNA levels of three specific genes, succinate dehydrogenase A (SDHA), matrix metalloproteinase 13 (MMP13) and interleukin 6 (IL6), were quantified. SDHA is a “housekeeping gene”, which means that it is not regulated by the experimental conditions of the study and can therefore be used as an internal control for errors introduced during the experiment (Table 4).

	Forward Primer	Reverse Primer
SDHA	TGGGAACAAGAGGGCATCTG	CCACCACTGCATCAAATTCATG
IL6	ATAGGACTGGAGATGTCTGAGG	AGGCAACTGGACCAAGG
MMP13	TCAGGAAACCACCTCTCCAC	TGACGCCAACAATACGGTTA

Table 4: Primer sequences of target genes

At first, the cDNA samples were diluted with RNase-free water at a ratio of 1:5 to a required volume. Then, the Master Mix containing forward primer, reverse primer, SYBR green and RNAse-free water was prepared (Table 5). Afterwards, 24 µl of the master mix and 1 µl of a cDNA sample were pipetted into a fast optical 96-well reaction plate by Applied Biosystems. Each cDNA sample was analyzed in duplicates. Moreover, a negative control of each target gene was included to exclude contamination. After that, the plate was sealed with an adhesive film and centrifuged for 1 min to reduce air bubbles. Finally, it was placed into the QuantStudio 3 (Applied Biosystems) and the program with a predefined temperature profile was started (Table 6). All these steps were performed on ice preventing denaturation.

Component	Volume (µl)
Forward Primer	0.5
Reverse Primer	0.5
SYBR Green	12.5
H_2O	10.5
Total per Reaction	24

Table 5: Master Mix components for RT-qPCR

Cycle	Step	Temperature	Time
Holding Stage	Step 1	95°C	10 min
PCR Stage	Step1	95°C	15 s
	Step 2	55°C	1 min
	Step 3	72°C	1 min
Melt Curve Stage	Step 1	95°C	15 sec
	Step 2	55°C to 95°C	+1°C/min
	Step 3	95°C	15 sec

Table 6: Temperature profile of RT-qPCR

There are three major steps involved in a PCR, which repeat for 30-40 cycles. These steps - denaturation, annealing and elongation - take place at different temperatures.

- Denaturation (95°C): separation of double-stranded DNA into single-stranded DNA
- Annealing (55°C): the primers bind at the single- stranded DNA
- Elongation (72°C): DNA-polymerase starts coping the template

For relative quantification of DNA-based fluorescence, a threshold has to be set the closest to the background signal. The cycle threshold (C_T) of a reaction is the number of cycles where the fluorescence can be detected above the threshold. ΔC_T-method was used to calculate the difference between the C_T values of the treated samples and the control samples. This difference was related to the housekeeping gene SDHA.

2.6. In-Cell Western

In-Cell Western (ICW) is a simple quantitative immunofluorescence assay, which is performed in a 96-well plate. This combination of Western blot and ELISA allows a quantification of intracellular signaling in whole cells.

At first, 15,000 chondrocytes or synoviocytes were seeded in a 96-well plate. It was important to reach a decent confluency to obtain consistent fluorescent signals afterwards. Starved cells were treated with 500 µM DP*, NaHS, D-Penicillamin and 10 ng/ml IL-1β (time points dependent on experiments). After this, cells were washed once with PBS and fixed with methanol (-20°C) for 10 min. After discarding methanol, cells were washed 3 times with PBS before unspecific binding sites were blocked with 50 µl blocking buffer (LI-COR Odyssey blocking buffer) for 90 min on a shaker. Then, primary antibodies were diluted with blocking buffer and 50 µl per well were added to the cells and incubated overnight at 4°C. On the next day, cells were washed 5 times with PBS (+ 0.1% Tween 20). Afterwards, the secondary antibodies diluted in Tween 20 and blocking buffer were added to the cell layers and incubated again for 1 h under light protection at RT. Then, 5 washing steps with PBS (+0.1% Tween 20) were done. Finally, after drying the plate, it was placed into the Odyssey CLx Infrared Imaging System (LI-COR Biosciences) and the protein expression levels were analyzed.

2.7. Detection of H_2S with WSP-1

For detection of H_2S in cell medium and inside cells, we used the H_2S fluorescence probe WSP-1 (Washington State Probe-1). WSP-1 reacts with H_2S and forms a fluorophore which has excitation and emission maxima of 465 and 515 nm, respectively. The fluorescence was detected with FLUOstar optima microplate-reader. At first, we performed preliminary tests with different concentrations (50-500 µM) of NaHS in full medium in a 96-well plate. The samples were incubated at 37°C for 5, 15, 30 and 60 min after which they were incubated with 50 µM WSP-1 at 37°C for 20, 10 and 5 min in the dark. After receiving the results, we could establish the best concentration and time of treatment for DP*. Therefore, we incubated 100 µM DP* for 5, 15, 30 and 60 min in full medium, followed by 10 min incubation with WSP-1. It is suggested that DP* as a prodrug can be activated by the

presence of free thiols to release H_2S. So we also incubated 100 µM DP* in the presence of 1 mM, 500 µM and 100 µM Glutathione or Cysteine in full medium for 10 min before we added 50 µM WSP-1 for 10 min.

2.8. Statistics

Statistical analyses were performed using SPSS 24.0. Normal distribution of the data was confirmed using the Shapiro-Wilk test. Statistical significance of the data was delineated using ANOVA with Dunnett-T post hoc test. The level of significance was set to $p<0.05$.

3. Results

3.1. Release of H_2S from NaHS and DP* in cell medium

To detect the release of H_2S from the substances NaHS and DP* in full medium, we used the fluorescence probe WSP-1. At first, we incubated various concentrations of NaHS in full medium with 50 µM WSP-1 for 10 min. Figure 14 (A) indicates the marked increase in fluorescence intensity in correlation with the concentration. These results suggest that NaHS can release H_2S immediately in cell medium. After this we analyzed the substance DP* in full medium (Figure 14 (B)). Following incubation of 100 µM DP* in medium for various time periods (5 min, 15 min, 30 min and 60 min), 50 µM WSP-1 were added and incubated for another 10 min. Dissolved in full medium, DP* was stable for at least 60 min, evidenced by little change of H_2S fluorescence intensity during the incubation time (Figure 14 (B), n=2).

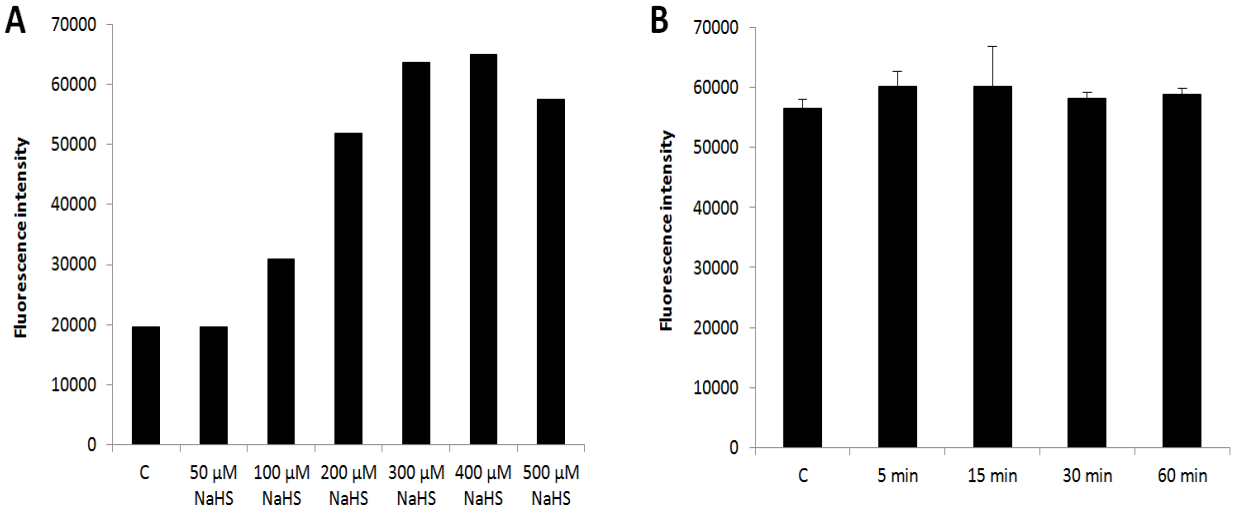


Figure 14: H_2S release from NaHS and DP* A: Different concentrations of NaHS were added to full medium in a 96-well plate and H_2S-induced fluorescence was observed after 10 min incubation with 50 µM WSP-1**. B:** 100 µM DP* were added to full medium for 5, 15, 30 and 60 min. Following 10 min incubation with WSP-1, the H_2S-induced fluorescence was detected.

3.2. Release of H_2S from DP* in presence of GSH and cysteine

After confirming the stability of DP* in cell culture medium, the combination of DP* with glutathione (GSH) or cysteine was tested. Therefore, we incubated 100 µM DP* in full medium for 10 min in presence/absence of GSH or cysteine. Then, 50 µM WSP-1 were added and incubated for another 10 min. As shown in Figure 15, a significant increase of fluorescence intensity generated by WSP-1 was observed after 10 min in presence of GSH or cysteine (*$p<0.05$, ANOVA, Dunnett post hoc-test, n=2). In comparison to the results shown in Figure 14 (B), this result indicates that DP* has the ability to dose-dependently release H_2S in presence of free thiols, such as GSH or cysteine.

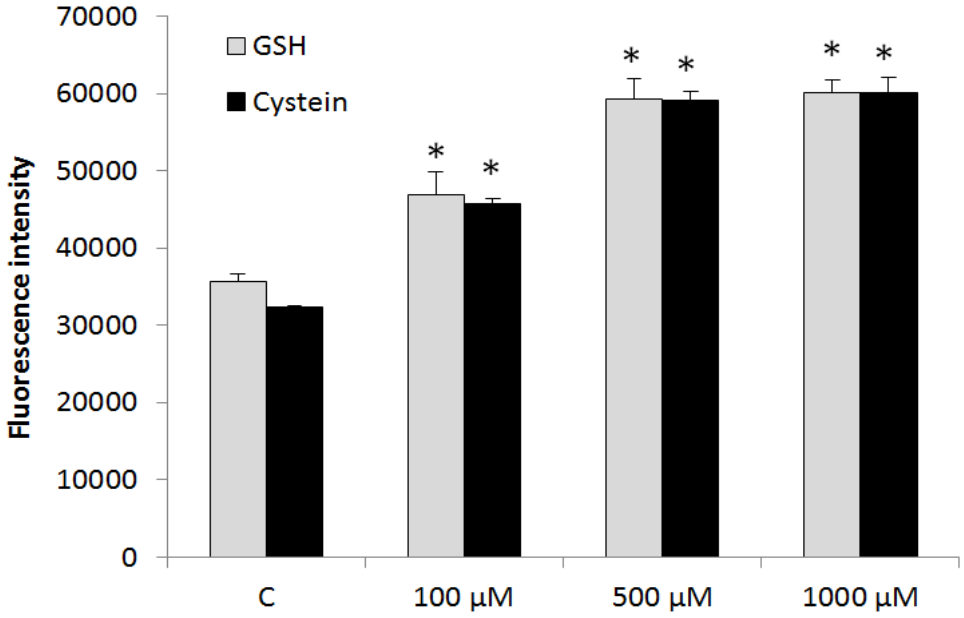


Figure 15: Dose-dependent release of H_2S in presence of GSH or cysteine. 100 µM DP*were incubated with three different concentrations (100 µM, 500 µM and 1000 µM) of GSH or cysteine for 10 min followed by an incubation with 50 µM WSP-1 for another 10 min. After the treatment the fluorescence intensity was measured. (*$p<0.05$ significant alteration compared to the control group)

3.3. Analysis of cell viability

To assess the cytotoxicity and to determine the maximum concentration of NaHS and DP* tolerated by OA chondrocytes (n=3 patients) and synoviocytes (n=3 patients), a MTT assay was performed. The cells were treated with 5 mM, 1 mM, 500 µM, 100 µM and 50 µM of NaHS or DP* for 24 and 48 hours.

3.3.1. Effect of NaHS and DP* on the metabolic cell activity on OA chondrocytes and OA synoviocytes

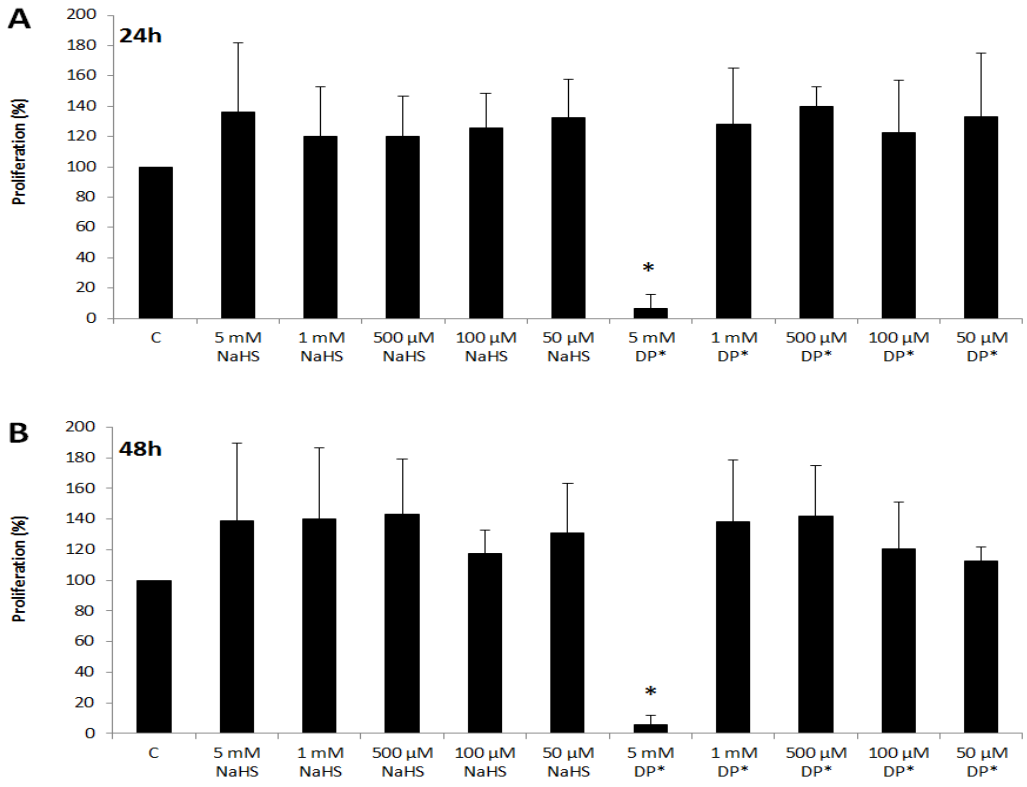


Figure 16: Effects of NaHS and DP* on the metabolic cell activity in OA chondrocytes. OA chondrocytes (n=3 patients) were treated for 24 h (A) and 48 h (B) with different concentrations of NaHS and DP* (5 mM, 1 mM, 500 µM, 100 µM and 50 µM) and compared to an untreated control (C). (*$p<0.05$: significant alteration compared to the control which was set to 100 %)

Figure 16 (A-B) indicates the impact of five different concentrations of NaHS and DP* on the metabolic activity of OA chondrocytes and OA synoviocytes, respectively. In general, most of the applied concentrations were well tolerated. However, the highest concentration (5 mM)

of DP* induced a statistically significant decrease of metabolic activity in OA chondrocytes after 24 h (A) and 48h (B) treatment (Figure 16 (A,B), *p<0.05, ANOVA, Dunnett post hoc test, n=3 patients).

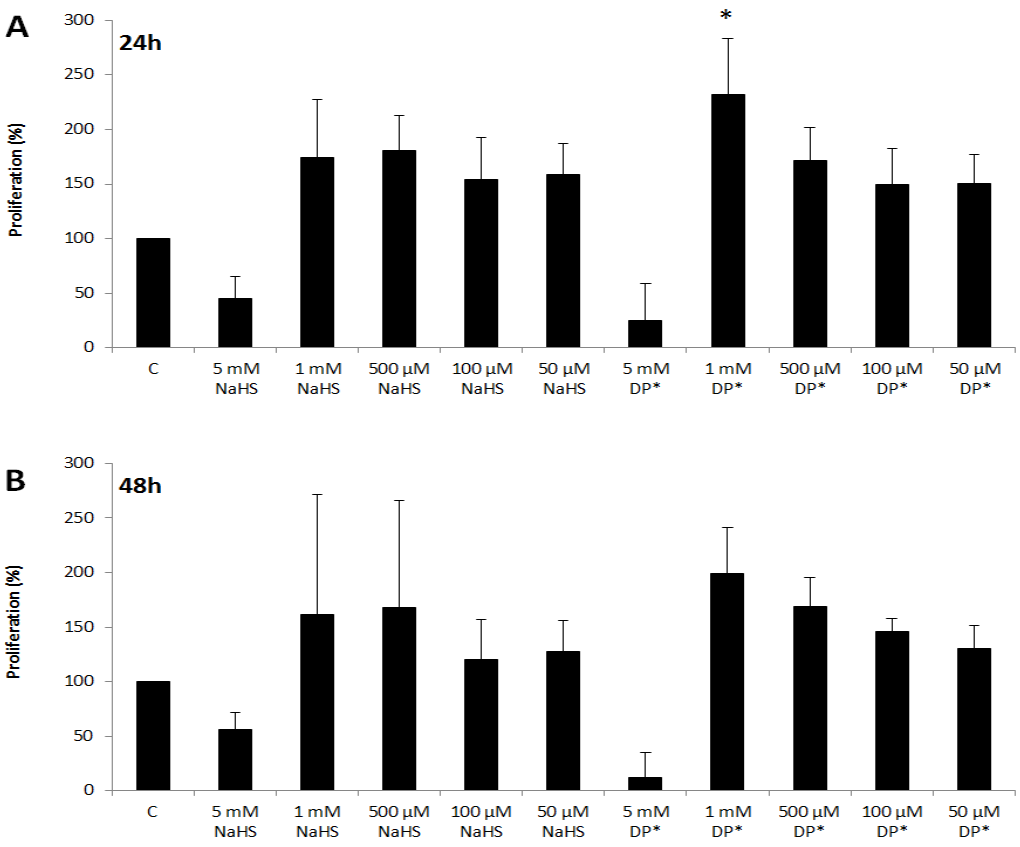


Figure 17: Effects of NaHS and DP* on the metabolic cell activity in OA synoviocytes. OA synoviocytes (n=3 patients) were treated for 24 h (A) and 48 h (B) with different concentrations of NaHS and DP* (5 mM, 1 mM, 500 µM, 100 µM and 50 µM) and compared to an untreated control (C). (*p<0.05: significant alteration compared to the control group which was set to 100 %)

Figure 17 (A-B) indicates that the treatment with 1 mM DP* for 24 h showed a significant increase in metabolic activity in OA synoviocytes (*p <0.05, ANOVA, Dunnett post hoc test, n=3 patients). Nevertheless, the highest concentration (5 mM) of both substances, NaHS and DP* showed a considerable impairment of cellular activity. Mainly, DP* affected the synoviocytes intensely, comparable to the results of OA chondrocytes. Although the results of DP* were not statistically significant, we observed in 2 out of 3 patients no metabolic activity at all. Moreover, microscopic analysis of synoviocytes treated with 5 mM DP*

showed an alteration of cell morphology in comparison with untreated cells. Based on these results, we used mainly the concentrations 500 µM, 100 µM and 50 µM for our following experiments with OA chondrocytes and OA synoviocytes.

3.4. Effects of NaHS and DP* on gene expression

RT-qPCR was performed to analyze the alteration of gene expression in response to treatments in OA chondrocytes and synoviocytes. The cells were treated with different concentrations of NaHS and DP* for 24 h. IL6 and MMP13 marker genes were determined in the following RT-qPCR experiments using SDHA as a reference gene.

3.4.1. Activity of NaHS and DP* in OA chondrocytes and OA synoviocytes

After the determination of an appropriate concentration for the cells, 50 µM, 100 µM and 500 µM NaHS and DP* were used to stimulate OA chondrocytes (n=3 patients). Figure 18 indicates that no significant effects neither on IL6 (A) nor on MMP13 (B) mRNA levels could be detected.

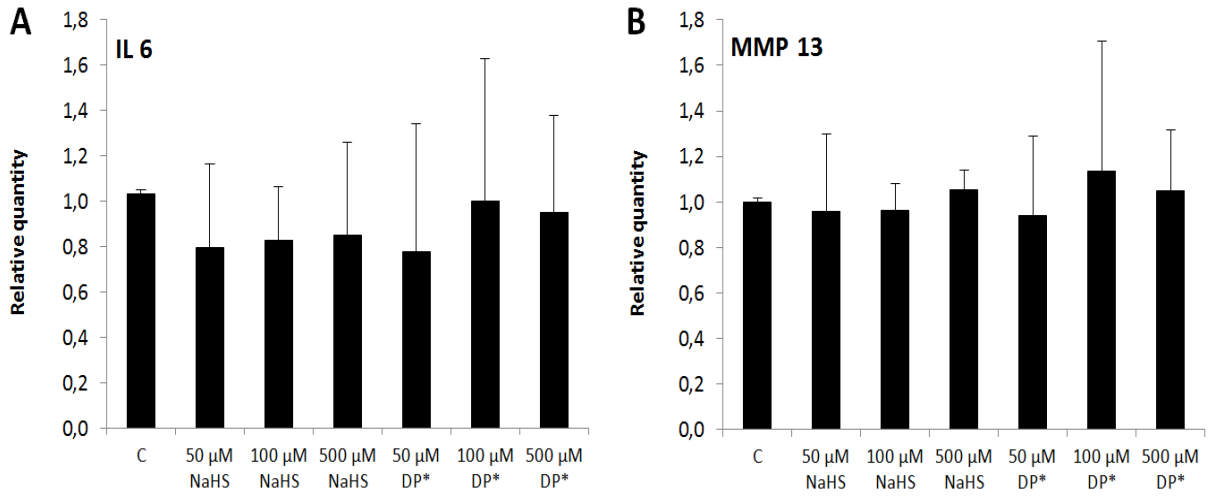


Figure 18: Dose-dependent effects of NaHS and DP* on IL6 (A) and MMP13 (B) mRNA levels in OA chondrocytes. OA chondrocytes (n= 3 patients) were treated for 24 h with different concentrations (50 µM,

100 µM and 500 µM) of NaHS and DP*. Untreated cells were cultured under the same conditions and used as reference. The mRNA levels of IL6 and MMP13 were quantified using RT-qPCR with SDHA as reference gene.

The same experimental set-up was performed in OA synoviocytes (n=3 patients), however, only the concentrations of 100 µM and 500 µM NaHS and DP* were tested. Figure 19 shows that also in these experiments, no significant effects of NaHS and DP* on mRNA expression levels could be observed.

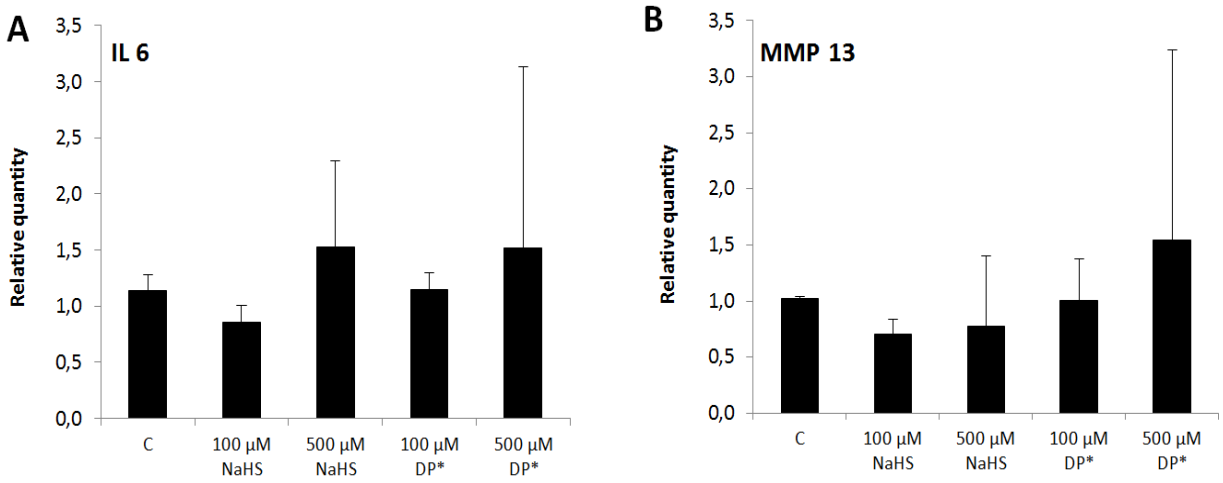


Figure 19: Dose-dependent effects of NaHS and DP* on IL6 (A) and MMP13 (B) mRNA levels in OA synoviocytes. OA synoviocytes (n= 3 patients) were treated for 24 h with different concentrations (100 µM and 500 µM) of NaHS and DP*. Untreated cells were cultured under the same conditions and used as reference. The mRNA levels of IL6 and MMP13 were quantified using RT-qPCR with SDHA as reference gene.

3.4.2. Activity of DP* in OA chondrocytes and OA synoviocytes treated with different concentrations of IL-1β

On the basis of our previous experiments, we pre-treated chondrocytes (Figure 20 (A), n=1 patient) and synoviocytes (Figure 20 (B), n=1 patient) with 500 µM DP* for 1 h and then added different concentrations of IL-1β. As a central mediator of pathogenesis in OA, IL-1β has the ability to induce an increase of IL6 mRNA levels. IL-1β concentrations of 10 ng/ml, 1 ng/ml, 0.5 ng/ml and 0.1 ng/ml were used. As presented in Figure 7, both in chondrocytes and synoviocytes the IL-1β stimulating effect was best detectable at 10 ng/ml, however DP* could decrease the IL-1β-induced IL6 mRNA levels in all used concentrations. To obtain significant and reproducible results, we used the IL-1β concentration of 10 ng/ml in the following experiments.

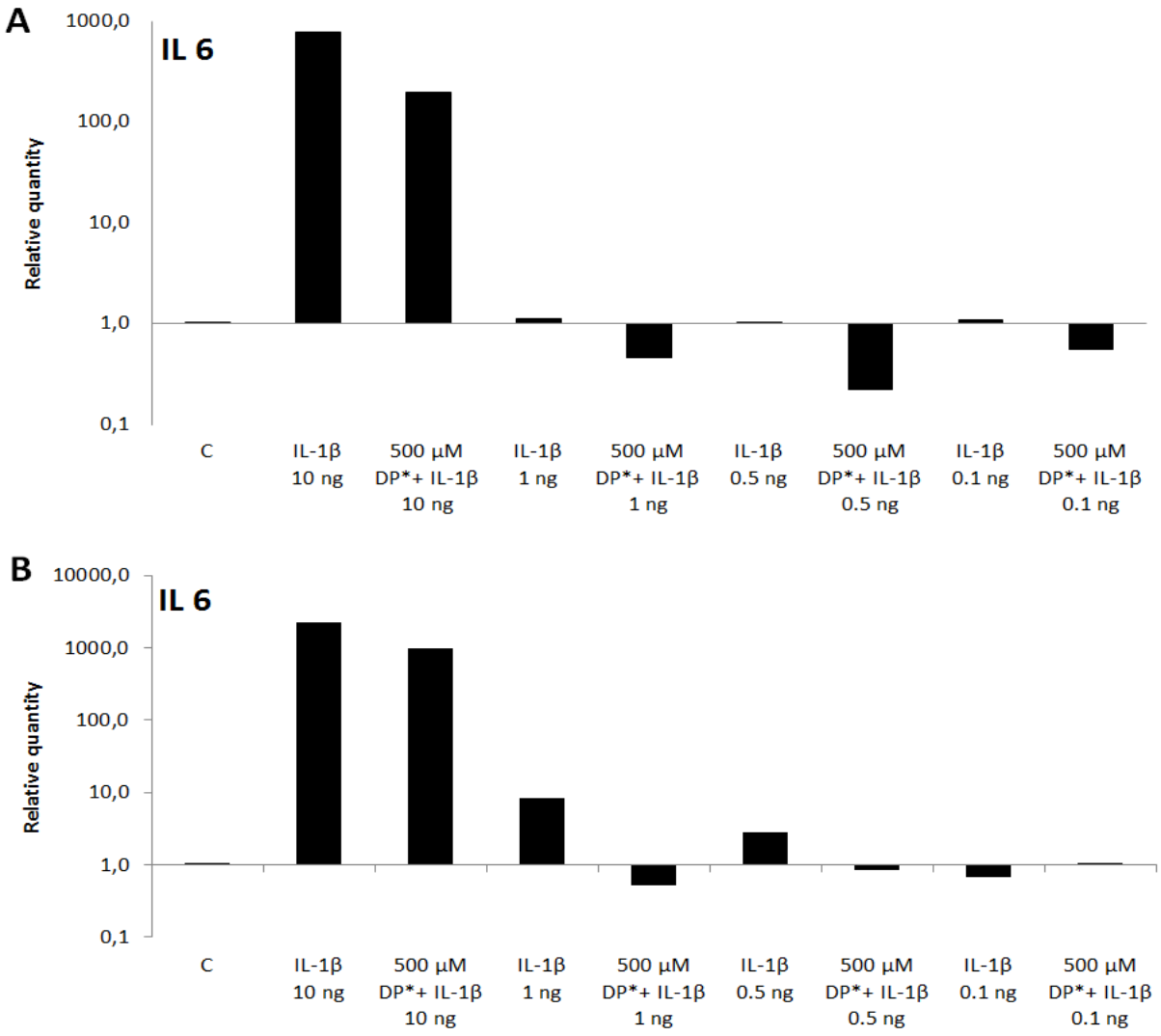


Figure 20: IL6 mRNA levels of OA chondrocytes with or without treatment with 500 µM DP* and after IL-1β-stimulation with different concentrations.OA chondrocytes (A, n= 1 patient) and OA synoviocytes (B, n=1 patient) were pre-treated for 1 h with 500 µM DP* followed by an incubation different concentrations (10, 1, 0.5 and 0,1 ng/ml) of IL-1β for 24 h. Untreated cells (C) and cells in absence of DP* were cultured under the same conditions and used as reference. The mRNA levels of IL6 were quantified using RT-qPCR with SDHA as reference gene.

3.4.3. Activity of 500 µM NaHS and DP* in OA chondrocytes treated with IL-1β at different time points

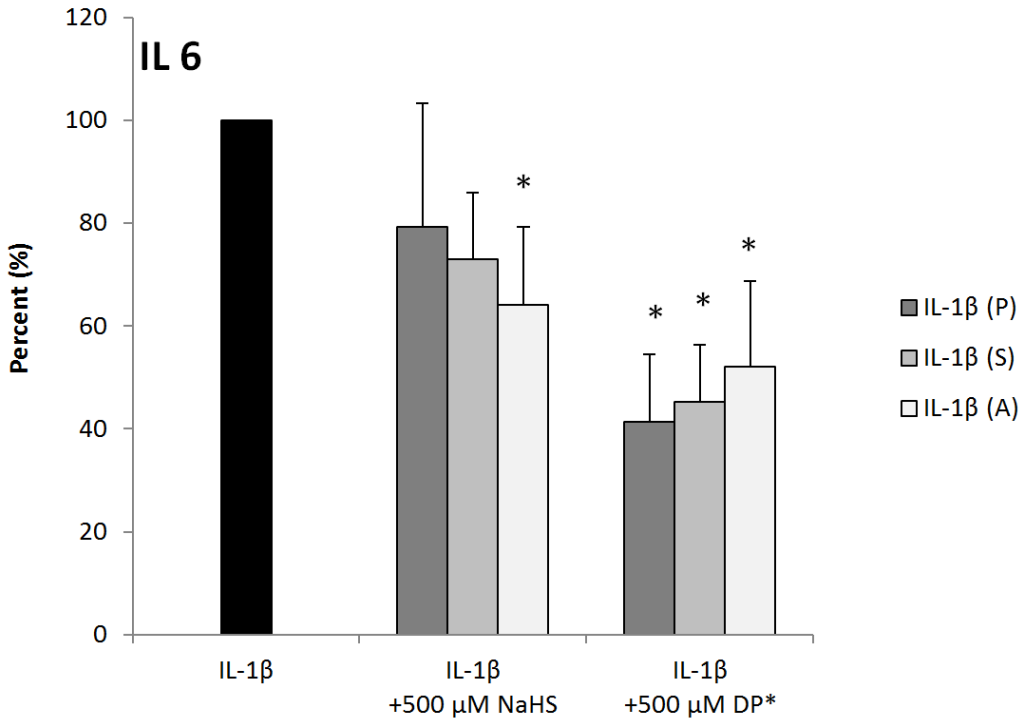


Figure 21: IL6 mRNA levels of OA chondrocytes after 24 h treatment with 500 µM NaHS or DP* and with IL-1β stimulation at different time points. OA chondrocytes (A, n= 4 patients) were treated for 24 h with 500 µM DP*. In addition, the cells were stimulated with 10 ng/ml IL-1β 1h before (P), simultaneous (S) or 1 h after (A) the treatment with DP*. Untreated cells (C) were cultured under the same conditions and used as reference. The mRNA levels of IL6 were quantified using RT-qPCR with SDHA as reference gene.

Figure 21 indicates that IL-1β-induced IL6 mRNA levels were significantly reduced by 500 µM DP* in primary chondrocytes, regardless of whether IL-1β was added 1 h before DP* (P), simultaneously with DP* (S), or 1 h after DP* (A) (*$p=0.001$, ANOVA, Dunnett post hoc test, n=4 patients). In contrast, the IL6 mRNA levels were significantly reduced by 500 µM NaHS (*p=0.13) only when IL-1β was added 1 h after NaHS. However, the reduction of NaHS was not as powerful as of DP* in all three conditions.

For this reason, we continued to stimulate the cells with IL-1β after 1 h of treatment with NaHS and DP* in following experiments.

3.4.4. Activity of 100 and 500 µM NaHS/DP* in IL-1β-stimulated OA chondrocytes and OA synoviocytes

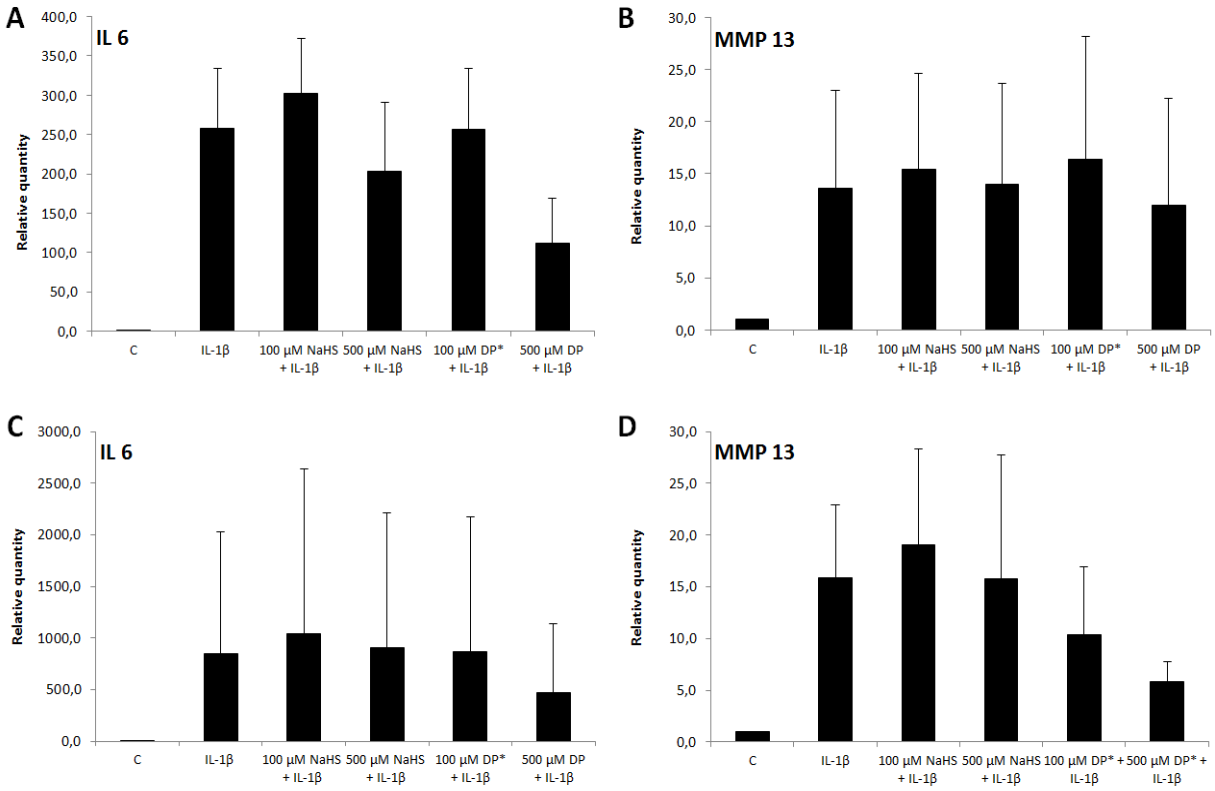


Figure 22: Effects of NaHS and DP* on IL-1β-induced IL6 (A, C) and MMP13 (B, D) mRNA levels in OA chondrocytes (A/B) and OA synoviocytes (C/D). OA chondrocytes (A, B, n=4 patients) and OA synoviocytes (C, D, n=3 patients) were treated for 24 h with different concentrations (100 µM and 500 µM) of NaHS or DP*. The cells were stimulated with 10 ng/ml IL-1β 1 h after the addition of DP*. Untreated cells (C) and cells in absence of DP* were cultured under the same conditions and used as reference. The mRNA levels of IL6 were quantified using RT-qPCR with SDHA as reference gene.

OA chondrocytes (Figure 22 (A, B), n=4 patients) and synoviocytes (Figure 22 (C, D), n=3 patients) were pre-treated with 100 µM and 500 µM NaHS or DP* for 1 h followed by the stimulation with 10 ng/ml IL-1β. Figure 9 indicates that no significant changes on IL6 and MMP13 mRNA levels were detectable. Nevertheless, the concentration of 500 µM DP* tend towards down-regulation especially of IL6 mRNA levels. Mainly in chondrocytes, 500 µM DP* could decrease the IL-1β-stimulated IL6 mRNA levels, however the statistical significance was not reached (Figure 22 (A), p=0,061, ANOVA, Dunnett post hoc test, n=4 patients).

3.4.5. Activity of 500 µM NaHS and DP* in IL-1β-treated OA chondrocytes and OA synoviocytes

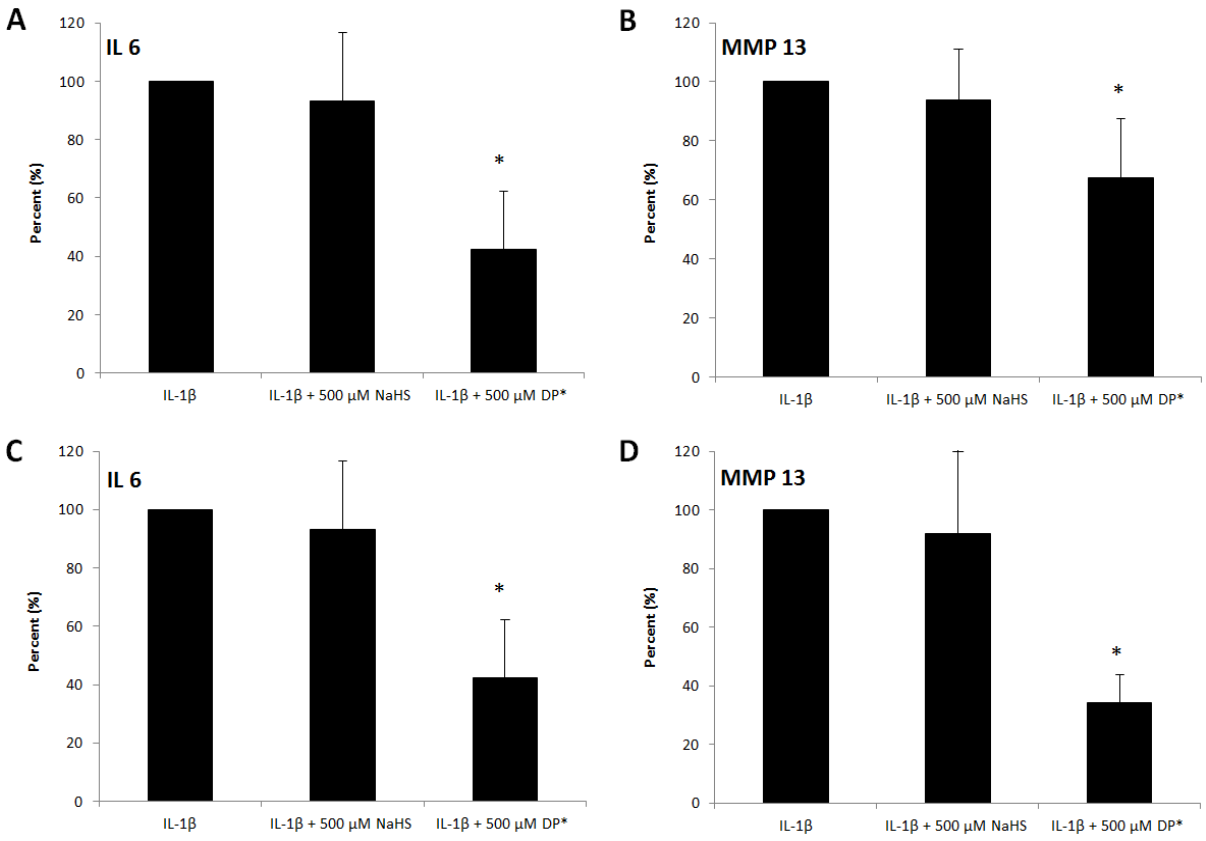


Figure 23: Effects of 500 µM NaHS and DP* on IL-1β-induced IL6 (A, C) and MMP13 (B, D) mRNA levels in OA chondrocytes (A, B) and OA synoviocytes (C, D). OA chondrocytes (A: n=9 patients (NaHS), n=14 patients (DP*), B: n=9 patients) and OA synoviocytes (C: n=3 patients (NaHS), n=6 patients (DP*), D: n= 3 patients) were treated for 24 h with 500 µM NaHS or DP*. The cells were stimulated with 10 ng/ml IL-1β 1 h after DP*. Untreated cells (C) and cells in absence of DP* were cultured under the same conditions and used as reference. The mRNA levels of IL6 were quantified using RT-qPCR with SDHA as reference gene. Results are expressed as the percentage of control, considering 100 % as the value of cells treated with IL-1β alone. (*$p<0.05$ compared with cells treated with IL-1β alone)

We repeated the treatment from our previous experiment with 500 µM DP* and NaHS on a larger patient group. Figures 23 indicates that the IL-1β-induced IL6 mRNA levels were significantly reduced by 500 µM DP* in OA chondrocytes (Figure 23 (A), n=14 patients) and synoviocytes (Figure 23 (C), n=6 patients) (*$p<0.05$, ANOVA, Dunnett post hoc test). Comparable effects were also observed with OA marker gene MMP13 (Figure 23 (B,D)). In contrast, 500 µM NaHS did not modify mRNA levels of IL6 and MMP13.

3.4.6. Activity of D-penicillamine in IL-1β-stimulated OA chondrocytes and OA synoviocytes

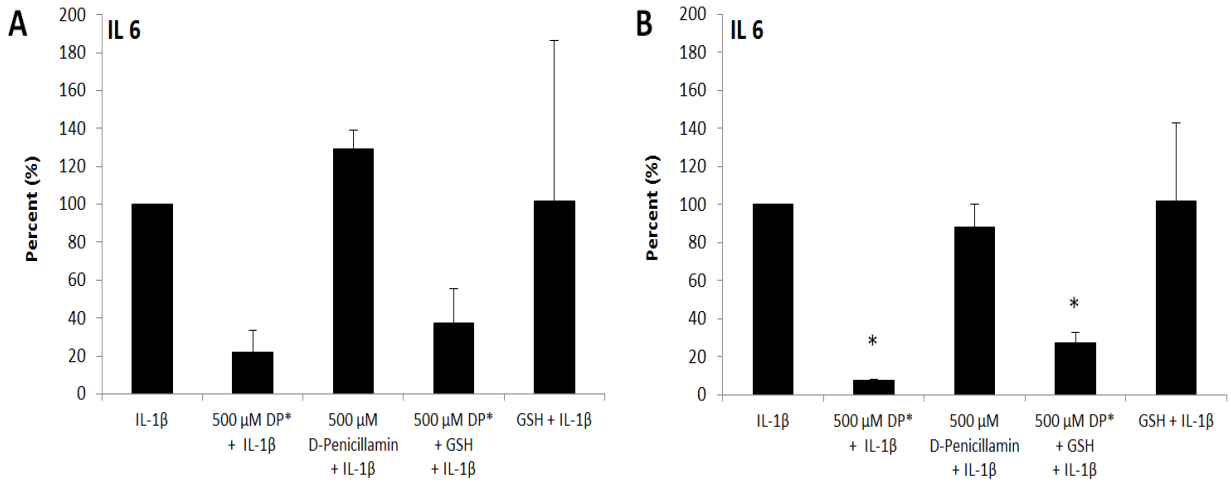


Figure 24: Effects of 500 µM DP* and D-penicillamine on IL-1β-induced IL6 mRNA levels in OA chondrocytes (A) and OA synoviocytes (B). OA chondrocytes (A, n=3 patients) and OA synoviocytes (B, n=2 patients) were treated for 24 h with 500 µM DP* or 500 µM D-penicillamine with and without 1 mM GSH. The cells were stimulated with 10 ng/ml IL-1β 1 h after the treatment with test substances. Untreated cells (C) and cells in absence of DP* were cultured under the same conditions and used as reference. The mRNA levels of IL6 were quantified using RT-qPCR with SDHA as reference gene. Results are expressed as the percentage of control, considering 100 % as the value of cells treated with IL-1β alone. (*$p<0.05$ compared with cells treated with IL-1β alone)

We treated OA chondrocytes (Figure 24 (A), n=3 patients) and synoviocytes (Figure 24 (B), n=2 patients) with 500 µM D-penicillamine, the precursor of DP*, and stimulated them afterwards with 10 ng/ml IL-1β. Figure 24 indicates that D-penicillamine and also GSH alone did not show any significant effect on IL6 mRNA levels when compared to IL-1β-stimulated chondrocytes and synoviocytes. Furthermore, in OA chondrocytes and synoviocytes, the inhibitory effect of DP* against IL-1β-induced IL6 expression was alleviated when DP* was added together with GSH. However, significant results were achieved only in OA synoviocytes (Figure 24 (B), *$p<0.05$, ANOVA, Dunnett post hoc test).

3.5. Effects of NaHS and DP* on cell signaling

In-cell western (ICW) analysis was performed to obtain a better understanding of the signaling pathways targeted by NaHS and DP* in OA chondrocytes. Therefore, in the following experiments we tested if either NaHS or DP* show any impact on the activation of NFκB or CREB pathways in OA chondrocytes (Figure 25) and OA synoviocytes (Figure 26).

OA chondrocytes of two different patients (n=2) were pre-treated with 500 µM NaHS, DP* or D-penicillamine for 1 h followed by an IL-1β- stimulation. One hour later, the cells were fixed and stained with antibodies raised against NF-κB or pNFκB (Figure 25).
Figure 12 indicates that in OA chondrocytes the IL-1β-stimulation increased NF-κB activation which was detectable due to an increase of fluorescence intensity. However, the substances NaHS, DP* and D-penicillamine did not show any significant decrease in NF-κB signaling after 1 h.

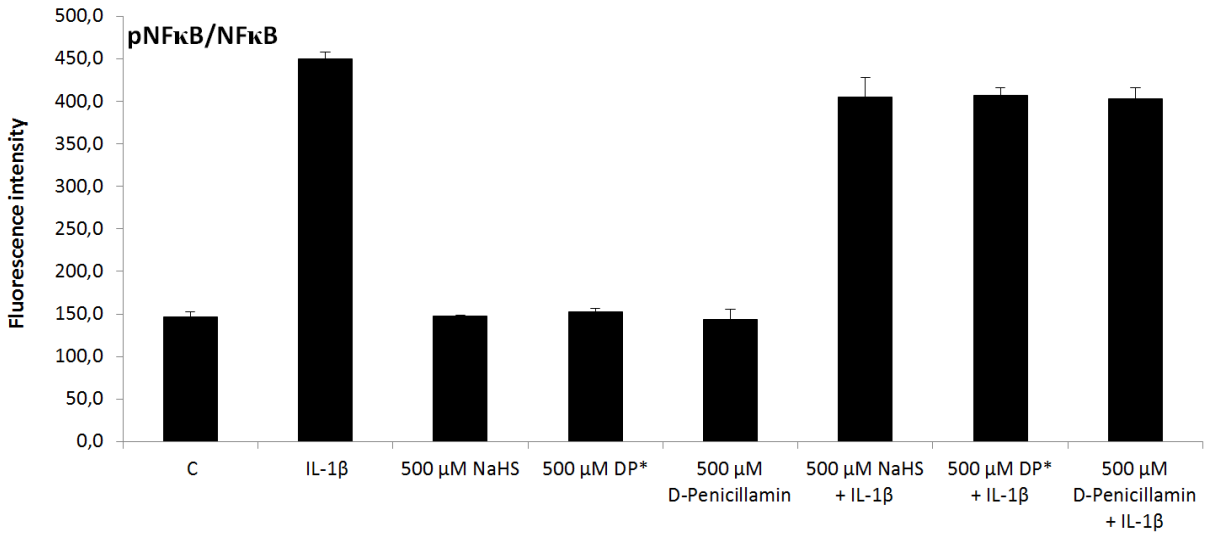


Figure 25: NF-κB activation in OA chondrocytes. OA chondrocytes (n=2 patients) were treated with 500 µM NaHS, DP* or D-penicillamine with and without IL-1β (10 ng/ml) stimulation. As reference, untreated cells and cells only with IL-1β stimulation were cultured under the same conditions. Fixed cells were incubated with antibodies against pNF-κB and NF-κB. After the treatment the fluorescence intensity was measured.

OA synoviocytes (n=2 patients) were treated with 500 µM NaHS or DP* for 3 h, 1 h and 15 min. Afterwards, the cells were fixed and stained with antibodies raised against pCREB and CREB. Figure 26 indicates that no significant alteration could be detected.

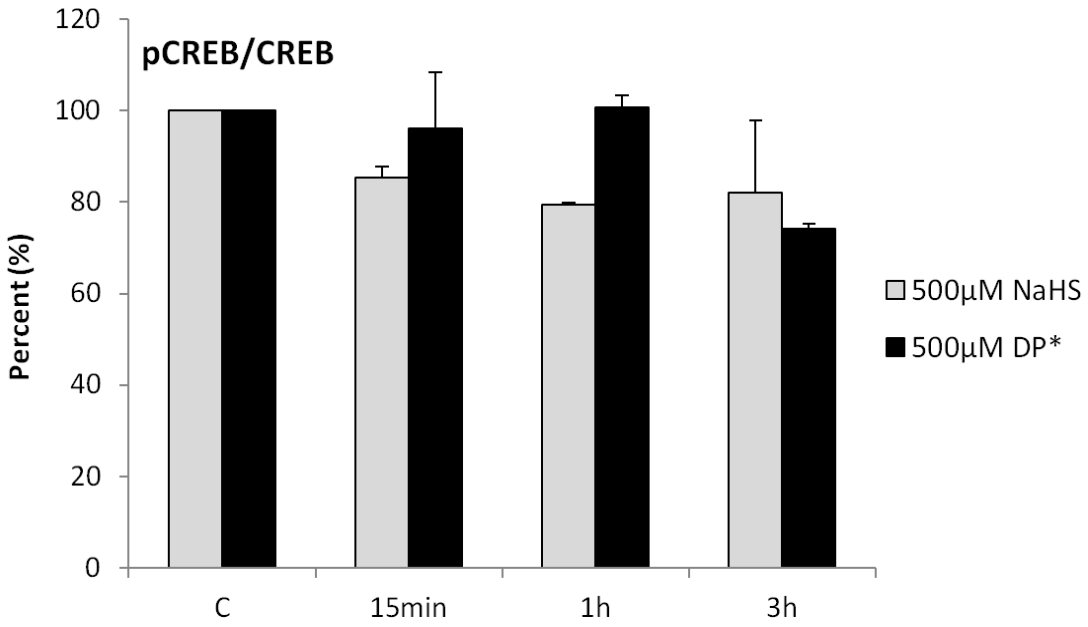


Figure 26: CREB activation in OA synoviocytes. OA synoviocytes (n=2 patients) were treated with 500 µM NaHS or DP* for 3 h, 1h and 15 min. As reference, untreated cells were cultured under the same conditions. Fixed cells were incubated with antibodies against pCREB and CREB. After the treatment the fluorescence intensity was measured. Results are expressed as the percentage of control, considering 100 % as the value of untreated cells.

4. Discussion

In recent studies, it was shown that H_2S plays an important role in inflammatory processes, where it is able to decrease mediators such as TNF-α - or IL-1β (Gemici and Wallace 2015). Furthermore, evidence has accumulated that oxidative stress participates in the development of inflammatory and degenerative joint diseases and that H_2S is able to protect human joint cells against oxidative injury (Fox et al. 2012). Hence, in recent years, H_2S-releasing compounds have received increased attention and have gradually been improved in various properties. Building on this knowledge, our aim was to evaluate the effect of a novel H_2S-releasing agent DP* in comparison to NaHS in human OA chondrocytes and OA synoviocytes.

The first step was to investigate and compare the H_2S release of DP* and NaHS in full medium. In accordance with the study from Zhao et al., (2014), NaHS released H_2S immediately in solution, which may lead to complications during in vitro experiments, due to loss of H_2S through volatilization. Therefore, sulfide salts may not be ideal substances for usage in clinical applications due to inadequate release kinetics and difficulty in concentration controlling. In contrast, the prodrug DP* showed a decent stability for about 1 h in full medium, indicated by the lack of detectable fluorescence intensity using H_2S sensitive fluorophore (Figure 14 (B)). Moreover, DP* was able to generate H_2S in presence of GSH or cysteine, which ensures a controlled and slower release. These findings are in agreement with Artaud and Galardon (2014), who found that DP* acts as an H_2S-free precursor for sulfur compounds in the absence of free thiols. Therefore, compared to a previous study (Zhao et al. 2014) on the substance GYYP4137 - another H_2S donor, which releases H_2S through simple hydrolysis - the donor DP* may be superior. H_2S release from GYY4137 is pH and temperature dependent, which could lead to low H_2S levels under physiological conditions. In comparison, DP* is able to generate H_2S in a similar manner to endogenously generated H_2S and shows therefore great potential for pharmaceutical applications.

To verify that NaHS and DP* have no cytotoxic effects on OA chondrocytes and OA synoviocytes at the used concentrations (5 mM, 1 mM, 500 µM, 100 µM and 50 µM), the metabolic cell activity was tested. The results indicated that the exposure of OA chondrocytes and OA synoviocytes to DP* at the concentration of 5 mM decreased cell viability. Moreover, the highest concentration (5 mM) of NaHS showed also an impairment of metabolic activity in OA synoviocytes. These findings were relevant to determine suitable

concentration ranges of both substances for ensuring experiments. Based on these data, the highest used concentration in this study was set to 500 µM for NaHS as well as for DP*.

It is well established that inflammatory mediators such as IL6 and MMP13 are responsible for cartilage destruction in OA (Goldring and Otero 2011). Thus, the next step was to evaluate the impact of H_2S-releasing compounds on the expression of these inflammatory mediators in OA chondrocytes and OA synoviocytes.

Firstly, a concentration-dependent treatment with NaHS and DP* was performed with both cell types, where no significant alteration of IL6 and MMP13 mRNA levels were detected. Therefore, we stimulated the cells additionally with 10 ng/ml IL-1β to mimic the pathophysiology of OA and to further stimulate the expression of IL6 and MMP13 mRNA levels. Of note, the concentration of 10 ng/ml IL-1β used for cell stimulation was markedly higher than the concentration range of IL-1β reported in the synovial fluid of OA patients, which has been reported to be 0.021-0.146 ng/ml (McNulty et al. 2013). However, in line with the literature (Weinmann et al. 2018) and based on our own experiments (Figure 20), showing that also lower concentrations of IL-1β lead to the same results, the applied concentration of 10 ng/ml in this study was chosen to induce a maximized and reproduceable inflammatory response in our experiments.

In agreement with Burguera et al., (2014), we demonstrated that the compound NaHS at concentrations of 500 µM could not significantly decrease IL-1β-stimulated IL6 and MMP13 mRNA levels in human articular chondrocytes. However, Burguera et al., (2014), revealed that 1000 µM NaHS were able to significantly reduce the increased mRNA levels in OA chondrocytes (n=4 patients, 5 ng/ml IL-1β, 48 h treatment). In comparison, we demonstrated that the prodrug DP* at the concentration of 500 µM was able to significantly attenuate the IL-1β-induced IL6 and MMP13 mRNA levels by more than a half. In all our experimental set-ups, we pre-treated the cells with NaHS or DP* for one hour before we added 10 ng/ml IL-1β. However, we found that regardless of whether we stimulated the cells with IL-1β 1 h before, simultaneously or 1 h after the treatment, the IL6 mRNA levels (Figure 21) could be decreased in all three conditions. In short, DP* as used here might not only reduce inflammation by regulating IL6 gene expression but could also minimize the degradation of cartilage leading to matrix breakdown by reducing MMP13 expression.

After exploring the effects of NaHS and DP* in OA chondrocytes and OA synoviocytes, we wanted to focus on the effect and the comparison of the precursor substance D-penicillamine and DP* in combination with GSH. Figure 24 showed that in comparison with DP*, D-penicillamine could not decrease IL-1β-induced IL6 mRNA levels. Furthermore, the combination of DP* with GSH alleviated the effect of DP* in OA chondrocytes and

synoviocytes. Together, these findings lead to the assumption that DP* is able to release H_2S inside the cell, where it unfolds its effect in presence of free thiols, such as Artaud and Galardon (2014) suggested. However, it may be also possible that the undissociated state of DP* or a possible conjugate with GSH might contribute the biological effect. Therefore, it is important to further investigate on how DP* unfolds its effects in vitro and in vivo.

In an attempt to elucidate the molecular mechanisms responsible for the inhibitory effects of DP* on inflammatory mediators, we further conducted In-Cell Western (ICW) experiments. Ha et al., (2015) described that IL-1β is able to activate the NF-κB pathway (based on Western blot experiments) in OA chondrocytes, which can be reduced by exogenous H_2S-donors. Therefore, ICW was performed to investigate if either NaHS or DP* show any impact on NF-κB signaling in OA chondrocytes. Interestingly, Figure 25 indicates that NaHS and DP* at the concentration of 500 µM could not decrease IL-1β induced NF-κB signaling. However, the fact that this experiment was conducted only on two patients and at one time-point, might explain the difference between our results and the literature.

Yang et al. (2014) revealed that an administration of H_2S could alleviate withdrawal symptoms of morphine and heroin addicted persons due to AC/cAMP/p-CREB pathway suppression. Once again, ICW was performed to investigate the effect of NaHS and DP* in CREB pathway of OA synoviocytes. Both substances NaHS and DP* tend towards down-regulation of CREB-pathway after 3 h. However, no significant data could be received due to the limited patient number (n=2) used for this experiment.

It should be briefly mentioned that to the best of our knowledge, studies investigating the effect of the H_2S-releasing compound DP* in articular cartilage have not been performed yet. Therefore, this study represents a first step towards the evaluation of DP* in human OA chondrocytes and OA synoviocytes. Results obtained so far suggest mainly an anti-inflammatory effect of DP* which might protect extracellular matrix from cytokine-induced degradation. However, more experiments, such as ICW or Western blot, are needed to further explore the DP*-induced gene expression pattern and to identify the responsible signaling pathways. Moreover, regarding our ICW experiments, further experiments with an increased number of patients are needed to achieve statistical significance. In addition, any results obtained in vitro might not directly translate to an in vivo situation. Therefore, future in vivo studies using disease models and human subjects will be mandatory to verify the therapeutic potential of DP*.

To sum up, our results show that, compared to NaHS, DP* decreases important functional disease markers in OA chondrocytes and OA synoviocytes representing a novel opportunity for therapeutic intervention in OA.

5. Conclusion

In summary, this study suggests that H_2S-releasing compounds might be effective in reducing cartilage inflammation and degeneration during the progression of OA. We compared and investigated the role of the two H_2S-releasing substances DP* and NaHS in primary OA chondrocytes and OA synoviocytes. Firstly, experiments with a fluorescence probe designed to detect H_2S revealed that NaHS released H_2S immediately in solution, whereas DP* provided prolonged stability. We demonstrate that DP* could only be activated in presence of GSH or cysteine, indicating that DP* might be a promising new H_2S-releasing agent with a controllable H_2S release. Furthermore, RT-qPCR experiments showed that the novel substance DP* could significantly decrease IL-1β-stimulated inflammatory mediators at mRNA levels in OA chondrocytes and OA synoviocytes. In comparison, the sulfide salt NaHS and D-penicillamine - the precursor of DP*- did not show any beneficial effects towards IL-1β-induced IL6 and MMP13 gene expression. In addition, DP* in combination with free thiols such as GSH alleviated the effect of DP^*, which might confirm the assumption that DP* is able to unfold its effect inside the cell. Future studies should further investigate the H_2S-releasing and molecular effects of DP* in OA chondrocytes and OA synoviocytes. Improved understanding of the prodrug DP* in OA might lead to the development of novel therapeutic and prophylactic therapy of OA.

6. References

ARTAUD I AND GALARDON E. 2014. A persulfide analogue of the nitrosothiol SNAP: formation, characterization and reactivity. Chembiochem 15: 2361-2364.

BROSSEAU L, YONGE KA, WELCH V, MARCHAND S, JUDD M, WELLS GA AND TUGWELL P. 2003. Thermotherapy for treatment of osteoarthritis. Cochrane Database of Systematic Reviews.

BURGUERA EF, VELA-ANERO Á, MAGALHÃES J, MEIJIDE-FAÍLDE R AND BLANCO FJ. 2014. Effect of hydrogen sulfide sources on inflammation and catabolic markers on interleukin 1β-stimulated human articular chondrocytes. Osteoarthritis and Cartilage 22: 1026-1035.

FOX B, SCHANTZ J-T, HAIGH R, WOOD ME, MOORE PK, VINER N, SPENCER JPE, WINYARD PG AND WHITEMAN M. 2012. Inducible hydrogen sulfide synthesis in chondrocytes and mesenchymal progenitor cells: is H2S a novel cytoprotective mediator in the inflamed joint? Journal of cellular and molecular medicine 16: 896-910.

GADALLA MM AND SNYDER SH. 2010. Hydrogen sulfide as a gasotransmitter. Journal of neurochemistry 113: 14-26.

GEMICI B AND WALLACE JL 2015. Chapter Nine - Anti-inflammatory and Cytoprotective Properties of Hydrogen Sulfide. In*:* CADENAS, E AND PACKER, L (Eds.) Methods in Enzymology: Academic Press, p. 169-193.

GLYN-JONES S, PALMER AJR, AGRICOLA R, PRICE AJ, VINCENT TL, WEINANS H AND CARR AJ. 2015. Osteoarthritis. The Lancet 386: 376-387.

GOLDRING MB AND OTERO M. 2011. Inflammation in osteoarthritis. Current opinion in rheumatology 23: 471-478.

HA C, TIAN S, SUN K, WANG D, LV J AND WANG Y. 2015. Hydrogen sulfide attenuates IL-1beta-induced inflammatory signaling and dysfunction of osteoarthritic chondrocytes. Int J Mol Med 35: 1657-1666.

HAQ I, MURPHY E AND DACRE J. 2003. Osteoarthritis. Postgraduate Medical Journal 79: 377-383.

HUNTER DJ AND FELSON DT. 2006. Osteoarthritis. BMJ 332: 639-642.

KATZ JN, EARP BE AND GOMOLL AH. 2010. Surgical management of osteoarthritis. Arthritis care & research 62: 1220-1228.

KOLLURU GK, SHEN X, BIR SC AND KEVIL CG. 2013. Hydrogen sulfide chemical biology: pathophysiological roles and detection. Nitric Oxide 35: 5-20.

MATHIESSEN A AND CONAGHAN PG. 2017. Synovitis in osteoarthritis: current understanding with therapeutic implications. Arthritis research & therapy 19: 18-18.

MCNULTY AL, ROTHFUSZ NE, LEDDY HA AND GUILAK F. 2013. Synovial fluid concentrations and relative potency of interleukin-1 alpha and beta in cartilage and meniscus degradation. Journal of orthopaedic research : official publication of the Orthopaedic Research Society 31: 1039-1045.

PAGE CJ, HINMAN RS AND BENNELL KL. 2011. Physiotherapy management of knee osteoarthritis. Int J Rheum Dis 14: 145-151.

PAUL BD AND SNYDER SH. 2012. H2S signalling through protein sulfhydration and beyond. Nature Reviews Molecular Cell Biology 13: 499.

SHARMA AR, JAGGA S, LEE SS AND NAM JS. 2013. Interplay between cartilage and subchondral bone contributing to pathogenesis of osteoarthritis. Int J Mol Sci 14: 19805-19830.

SHEN X, KOLLURU GK, YUAN S AND KEVIL CG. 2015. Measurement of H2S in vivo and in vitro by the monobromobimane method. Methods in enzymology 554: 31-45.

VANDIVER MS AND SNYDER SH. 2012. Hydrogen sulfide: a gasotransmitter of clinical relevance. Journal of molecular medicine (Berlin, Germany) 90: 255-263.

WALLACE JL AND WANG R. 2015. Hydrogen sulfide-based therapeutics: exploiting a unique but ubiquitous gasotransmitter. Nature Reviews Drug Discovery 14: 329.

WEINMANN D, MUELLER M, WALZER SM, HOBUSCH GM, LASS R, GAHLEITNER C, VIERNSTEIN H, WINDHAGER R AND TOEGEL S. 2018. Brazilin blocks catabolic processes in human osteoarthritic chondrocytes via inhibition of NFKB1/p50. J Orthop Res 36: 2431-2438.

WHITEMAN M AND WINYARD PG. 2011. Hydrogen sulfide and inflammation: the good, the bad, the ugly and the promising. Expert Review of Clinical Pharmacology 4: 13-32.

YANG H-Y, WU Z-Y, WOOD M, WHITEMAN M AND BIAN J-S. 2014. Hydrogen sulfide attenuates opioid dependence by suppression of adenylate cyclase/cAMP pathway. Antioxidants & redox signaling 20: 31-41.

ZHANG D, DU J, TANG C, HUANG Y AND JIN H. 2017. H(2)S-Induced Sulfhydration: Biological Function and Detection Methodology. Frontiers in pharmacology 8: 608-608.

ZHANG W, OUYANG H, DASS CR AND XU J. 2016. Current research on pharmacologic and regenerative therapies for osteoarthritis. Bone research 4: 15040-15040.

ZHAO Y, BIGGS TD AND XIAN M. 2014. Hydrogen sulfide (H2S) releasing agents: chemistry and biological applications. Chemical communications (Cambridge, England) 50: 11788-11805.

ZHENG Y, YU B, DE LA CRUZ LK, ROY CHOUDHURY M, ANIFOWOSE A AND WANG B. 2018. Toward Hydrogen Sulfide Based Therapeutics: Critical Drug Delivery and Developability Issues. Med Res Rev 38: 57-100.

7. Appendix

7.1. Abbreviations

ADAMTS	a disintegrin and metalloproteinase with thrombospondin-like domains
cDNA	complementary deoxyribonucleic acid
C	control
CAT	cysteine aminotransferase
CBS	cystathionine β-synthase
CO	carbon monoxide
COX	cyclooxygenase
CREB	cyclic AMP response element binding protein
CSE	cystathionine γ-lyase
C_T	cycle threshold
Cys	cysteine
DMEM	Dulbecco´s Modified Eagle´s Medium
ECM	extracellular matrix
EDTA	ethylenediaminetetra-acetic acid
EZ4U	easy for you
FBS	fetal bovine serum
GAG	glucosaminoglycan
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GSH	glutathion
H_2S	hydrogen sulfide
ICW	in cell western
IL	Interleukin
K_{ATP}	ATP-dependent potassium channel
MMP	matrix metalloproteinase

mRNA	messenger ribonucleic acid
Na_2S	sodium sulfide
NaHS	sodium hydrosulfide
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NO	nitric oxide
NSAIDS	nonsteroidal anti-inflammatory drugs
OA	osteoarthritis
P5P	pyridoxal-5´-phosphate
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Penstrep	penicillin/streptomycin
pKa	acid dissociation constant
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
RT-qPCR	quantitative real-time reverse transcription polymerase chain reaction
SDHA	succinate dehydrogenase complex, subunit A
TNF	tumor necrosis factor
UV-vis	Ultraviolet-visible spectroscopy
WSP-1	washington state probe-1

7.2. List of figures

7.3. List of tables