Unraveling the potential for the novel agent, VR23, and its use as an anti-inflammatory for both acute and chronic inflammatory conditions

by

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Abstract

Inflammatory conditions continue to be on the rise in Canada, due in part to the increase in aging population. Although effective in some cases, the anti-inflammatory drugs that are currently available have their own pitfalls, with toxic side effects and being non-selective in their mechanisms of action. In an attempt to develop an effective anti-inflammatory drug, I have characterized VR23, a novel 4-aminoquinoline derived sulfonyl hybrid compound. VR23 was initially developed in our laboratory as potentially an effective and safe anticancer agent. Previously, data obtained from an *in vivo* study for its anticancer effects raised a possibility that VR23 might also possess anti-inflammatory property. In a nutshell, data presented in this thesis confirm that the hypothesis is correct. In the study, I used both acute and chronic inflammatory models. In Chapter 1, I have shown that VR23 is able to effectively down-regulate pro-inflammatory cytokines comparably to dexamethasone, a well-known anti-inflammatory agent. Specifically, VR23 was able to down-regulate IL-6 with great sensitivity. In rheumatoid arthritis cell models of chronic inflammation, VR23 demonstrated superiority over the anti-rheumatic hydroxychloroquine in its ability to regulate pro-inflammatory cytokines. In Chapter 2, I demonstrated VR23's anti-inflammatory mechanism is likely through its prevention of the phosphorylation of STAT3, leading to a decrease in the production of its down-stream targets, IL-6 and MCP-1. Lastly, in Chapter 3 I describe the discovery that VR23 is rapidly metabolized into CPQ and DK23. CPQ is not an active compound with respect to its anti-inflammatory activity, indicating that it is a by-product of the VR23 detoxification process. On the contrary, DK23 possesses active anti-inflammatory property, as potent as VR23 at their respective IC_{50} concentrations. Data from an acute lung injury model showed that the anti-inflammatory activity of VR23 is comparable to that of dexamethasone, a well-known corticosteroid. Data obtained from the rheumatoid arthritis study showed that VR23 is much more superior to hydroxychloroquine, a commonly used anti-rheumatic drug. Overall, this study demonstrates the potential for the novel compound, VR23, to be used as a non-toxic specific anti-inflammatory drug to treat IL-6 driven conditions such as rheumatoid arthritis.

Keywords

Inflammatory conditions, novel anti-inflammatory, rheumatoid arthritis, pro-inflammatory cytokines, IL-6, STAT3, VR23, hydroxychloroquine

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1.0 Introduction

1.1 Acute inflammation

Inflammatory conditions remain prevalent in the population, claiming numerous lives each year. In Canada, acute inflammation caused by influenza and pneumonia is the 7th leading cause of death [1]. Acute inflammation occurs rapidly after a bodily injury or illness occurs [2]. Classical signs and symptoms of acute inflammation include pain, swelling (edema), redness, loss of function, and heat at the affected area [3]. Inflammation is a response to tissue injury caused by environmental agents, trauma, or infection [3]. The inflammatory response can be helpful in healing wounds and controlling infection, but also can be pathological in many chronic disease states when not properly regulated [3]. Acute inflammation as an initial response is generally non-specific, as it works as the first line of defense of the body against the potential invaders [4]. Three major events occur during the inflammatory response: increase in blood supply to the affected area, increase of capillary permeability, and leucocyte migration from the interstitial spaces to the site of inflammation or injury [5]. Within the cells recruited, the first responders are phagocytic immune cells. These cells work to detect bacterial cell components in order to become activated [6]. Upon activation, cells release pro-inflammatory cytokines such as Tumor Necrosis Factor (TNF-α), Interleukin (IL)-1, IL-6, IL-8 to recruit more immune cells to the site of infection [6]. Anti-inflammatory mediators such as IL-10 and TGF-β also are released to prevent the excessive unregulated secretion of pro-inflammatory cytokines [4]. Activation of phagocytic cells kills bacteria directly through engulfment and/or the secretion of oxygen free radicals [4]. In ideal conditions, the acute inflammatory response can eliminate the pathogen and then subside the symptoms [7, 8]. However, there are cases where the response may not be potent enough to clear the pathogen [7, 8]. This could lead to a stronger non-regulated inflammatory response that may act in a positive feedback loop furthering inflammation [7, 8]. The sustained inflammatory response and the persistence of high levels of pro-inflammatory cytokines can lead to septic shock, a sudden life-threatening condition [7, 8].

1.1.1 Current therapies for acute inflammation

Treatment of acute inflammation becomes necessary if the inflammatory reaction is severe or if the infection is entering the blood, leading to sepsis [9]. In some acute inflammatory infections, antibiotics or anti-fungal treatments will be prescribed [10, 11]. For treatment specific to inflammation, nonsteroidal anti-inflammatory drugs or corticosteroids are often used [12, 13].

1.1.1.1 NSAID

Nonsteroidal anti-inflammatory drugs (NSAIDs) are used to relieve pain, swelling, and fever, without affecting the cause of inflammation [14]. NSAIDs work by inhibiting the enzymes, cyclooxygenase-1 or -2, which are responsible for the synthesis of the biological mediators, prostaglandins [14]. Prostaglandins are lipid compounds and are active in blood clotting processes and are found in an increased amount in inflamed tissue, with pro-inflammatory properties [15].

NSAIDs while effective in treating acute inflammation, have associated risks with their use, including upper gastrointestinal bleeding and ulceration [14, 16]. In addition, COX-2 NSAIDs have been associated with an increased risk of cardiovascular events such as myocardial infarction [14, 16, 17].

1.1.1.2 Corticosteroids

Corticosteroids (or glucocorticoids) have been widely used for acute inflammation owing to their fast acting ability to combat inflammation [13]. Corticosteroid is a synthetic version of cortisol, a hormone produced by the adrenal gland [13]. Corticosteroids work to reduce inflammation by binding glucocorticoid receptors, leading to the suppression of multiple inflammatory genes [13]. At higher doses, corticosteroids have the ability to interact with DNA recognition sites to activate transcription of anti-inflammatory genes [13].

A drawback of corticosteroids is their immunosuppressive nature, which causes patients to be more susceptible to infections [18]. A common long-term side effect of corticosteroids is bone loss, leading to fractures [19]. In addition, the prolonged usage of corticosteroids can cause

adrenal glands to stop making cortisol, leading to dangerously low blood pressure if discontinued suddenly [18]. The long-term use of steroids can also lead to other adverse side effects including cardiovascular effects [20], ophthalmologic [21] and gastrointestinal effects [22].

1.2 Chronic Inflammation

Chronic inflammatory conditions are the most significant cause of death in the world [23]. The prevalence of diseases with chronic inflammation are expected to increase over the next 30 years in the United States, due mainly to the increase of the aging population. Chronic inflammatory conditions include stroke, chronic respiratory diseases, heart disorders, arthritis and joint diseases, cancer, obesity and diabetes.

Chronic inflammation is long-term inflammation, which may last for periods of months to years [24]. The perpetuation of acute inflammation can lead to chronic inflammatory conditions. In contrast to acute inflammation, in chronic inflammation there is the continued recruitment of monocytes, lymphocytes, and plasma cells to the site of inflammation [25]. The accumulation of monocytes contributes to inflammation through the formation of monocyte-derived macrophages [25]. Macrophages act as a critical element of the inflammatory response and their distinct populations of M1 and M2 macrophages work to regulate the inflammatory environment [25]. The recruited monocytes and monocyte-derived macrophages are critical in the persistence of chronic inflammation by yielding many active products, including proteases, chemotactic factors, reactive oxygen, growth-promoting factors, and cytokines [26]. The presence of lymphocytes and plasma cells form the immune reaction of antibody production and hypersensitivity response [26]. In some chronic inflammatory responses, the pro-inflammatory response by the immune cells can lead to tissue damage and fibrosis or granuloma formation [25].

1.3 Autoimmune disorders

Autoimmune disorders or diseases occur in about 3-5 % of the population in the United States, with women being affected more often than men [27, 28]. Autoimmune disorders/diseases are conditions that form from a dysregulated immune response, leading to the immune system

mistakenly attacking healthy cells, tissues, or organs [29]. The delicate balance of recognition of pathogens and avoiding self-attack becomes impaired in autoimmune conditions, leading to continued inflammation and immune activation [30]. Continuous immune activation without a specific infection is what occurs during the flares of autoimmune diseases [30]. Currently, there are about 80 different conditions involving various body parts including lungs, eyes, heart, skin, and joints [29]. The direct cause of autoimmune conditions is unknown; however, some have known genetic transmission or are triggered by infections or environmental factors. While signs and symptoms vary between conditions, some general symptoms are fatigue, low grade fever, general feeling of unwellness, muscle aches, and rash.

Autoimmune disorders range from being organ specific, by which antibodies and T cells react to self-antigens in a specific tissue, to systemic, which demonstrate reactivity against a specific antigen spread throughout the body [31]. Organ specific diseases show predominance in Th1 cytokines like IL-2 and IFN-γ, leading to an increase in effector responses. Cell mediated immune responses lead to cytotoxic T cell killing through cytokine or antibody release [31]. Organ specific autoimmune conditions often target the thyroid, stomach, pancreas, and adrenal glands [31]. Due to specific antigens expressed on certain organs, there are autoimmune disease for every organ in the body [32]. Systemic autoimmune conditions are characterized by high levels of Th2 cytokines, IL-4, IL-5, and IL-10, in addition to the presence of autoantibodies and immune complexes [31]. Systemic autoimmune disorders typically involve the skin, joints, and muscle tissue [31] .

While many autoimmune conditions can be classified by their organ specificity or systemic traits, some diseases such as multiple sclerosis demonstrate both components [31]. In addition, certain conditions involve the targeting of specific cell types rather than organs, leading to conditions like systemic lupus erythematosus (SLE) [32]. Regardless, if cells or organs are targeted, these diseases are a result of an antigen specific reaction [32].

1.3.1 Rheumatoid arthritis

Rheumatoid arthritis (RA) is the most common chronic autoimmune condition that leads to the damage of the cartilage and bone, making it the leading cause of disability worldwide [33].

Approximately one out of every 100 Canadians are affected by RA, with women being 2 to 3 times more likely to develop the condition than males [34]. It is estimated that RA affects about six million people in Canada, representing about 20% of the population [35]. While there are many different treatment options for RA, there is no cure [35]. It is estimated that by 2040, approximately nine million Canadians will be living with this chronic condition. Patients with RA often experience joint pain, stiffness and swelling, and joint damage can occur if left untreated [36]. The exact etiology of RA remains unknown, but it is believed that genetic factors combined with various environmental factors can lead to the occurrence of chronic inflammation [37].

Autoantibodies for RA have been identified as rheumatoid factor (RF) and anti-cyclic citrullinated peptides (anti-CCP) autoantibodies. Rheumatoid factor is an autoantibody that acts on the Fc region of IgG, and has been used as a diagnostic tool for RA [38, 39]. While positive RF does indicate more severe disease activity, it is not specific to RA and can be found in other autoimmune patients [40, 41]. The other autoantibody identified specific to RA is anti-cyclic citrullinated peptides [42]. Anti-CCP autoantibodies are created as a response to citrulline production due to inflammation and oxidative stress [42]. Anti-CCP autoantibodies are also used as a diagnostic tool, and in some studies have been found in patients prior to the development or onset of disease [43]. Since there are variable citrullinated autoantigens that ACPA recognizes, each autoantigen is only present in a particular subset of RA.

1.3.1.1 Histopathology

In healthy joints, synovial tissue is critical for providing lubrication and nutrients to the joint for normal functioning [44]. In RA, the synovial tissue within the joint capsule is primarily where the immune response occurs, leading to synovitis, the inflammation of the synovial membrane [45, 46]. This occurs when the synovial lining, typically two to three cells thick undergoes dramatic hyperplasia and increase to 10 to 15 cells thick [47]. This prominent histological change is the hyperplasia of the synovium (including fibroblasts and fibroblast like synoviocytes) leading to the formation of an invasive granulation tissue, known as the pannus (Fig. 0.1).

The pannus is similar to a tumor-like tissue as it allows immune cells to infiltrate it, giving them greater access to the cartilage and bone, furthering destruction [48]. Within the pannus tissue, there are CD4+ T cells, B cells, macrophage and fibroblast-like mesenchymal cells [49], macrophage-like cells, and many other inflammatory cells [49-51]. The pannus allows these inflammatory cells to be in closer proximity to the bone and cartilage, furthering destruction *via* their release of degradative enzymes and inflammatory cytokines such as IL-1β, IL-6, and TNF-α [52]. The ability of synovial fibroblast within the pannus to produce matrix metalloproteinases and cathepsins increases their capacity to contribute to joint destruction. Taken together, the inflammatory pannus in RA patients is critical in the pathogenesis of the inflammatory condition.

1.3.1.2 Propagation of RA

The propagation of RA is complex and involves the recruitment and activation of many immune cell types that lead to the inflammation and destruction of the joint. CD4+ T cells play a significant role in the cell mediated immune response and have been implicated as having a key role in RA, by responding to an arthritogenic antigen [53]. Upon activation, the CD4+ T cells can stimulate other inflammatory cell types in the synovial cavity such as monocytes, macrophages, and synovial fibroblast and secrete cytokines including IL-2, TNF, and IL-4 (Fig. 0.1) [54, 55]. Further, the CD4+ T cells can also induce B cells through contact bindings to produce immunoglobulins [55]. B cells can also be found in rheumatoid synovium as lymphoid aggregates and contribute to cytokine production and can act as antigen presenting cells to T cells [56, 57]. The T cells and B cells represent the immunological cell types of RA; however, the damage of bone and cartilage in RA is driven by effector cells and their pro-inflammatory mediators. Macrophages are critical drivers of inflammation in RA, as they produce many pro-inflammatory cytokines including TNF, IL-1, IL-6, MCP-1 and IL-8 [58, 59]. These pro-inflammatory cytokines will further stimulate cytokines and activate other nearby cell types such as fibroblasts and osteoclasts [60, 61]. The synovial fibroblasts have also been implicated as drivers of inflammation in RA, as they secrete cytokines such as IL-6, IL-8, and other destructive proteases and collagenases [62, 63].

Through dysregulated secretion of pro-angiogenic factors by synovial tissue macrophages and fibroblasts, angiogenesis occurs in RA patients [64]. The increase in blood flow to the area can propagate the inflammation by allowing destructive inflammatory cells to migrate to the area [64].

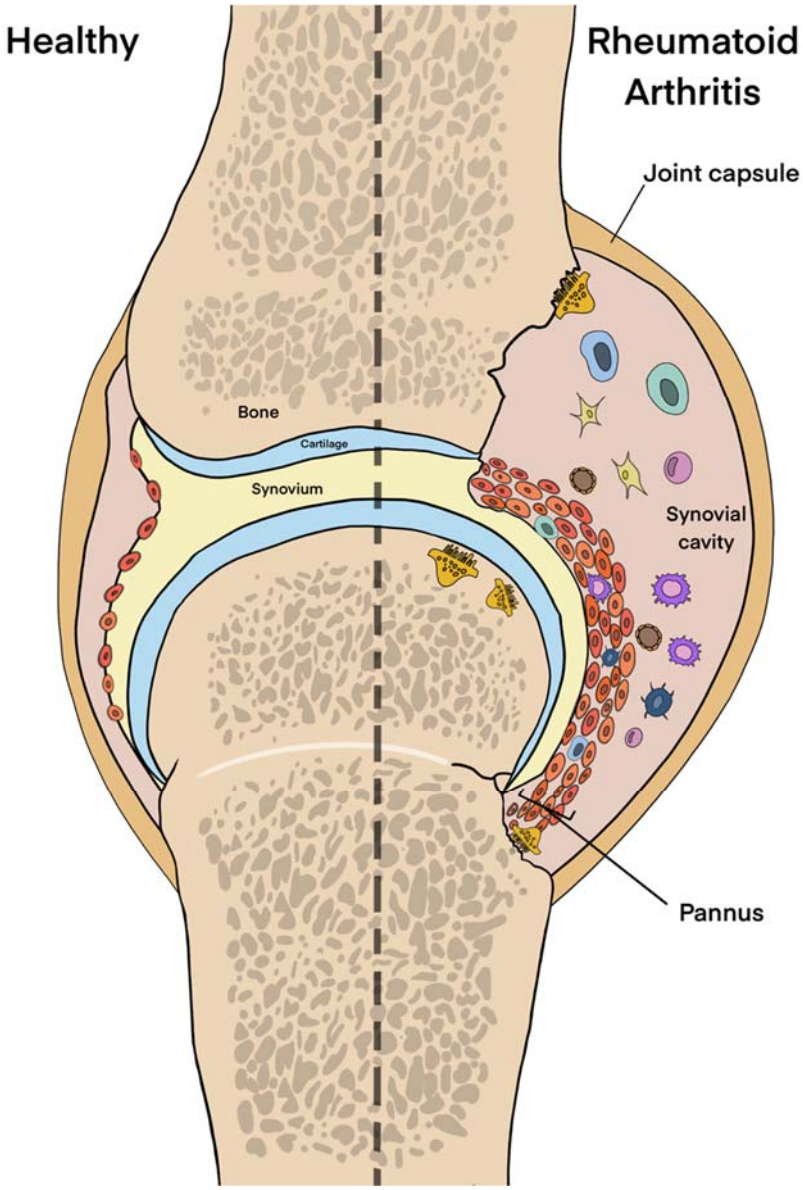

Figure 0.1. Pathogenesis of a rheumatoid arthritis joint compared to a healthy joint. In a healthy joint, the synovium within the joint capsule secretes lubricants that allow for the movement and maintenance of the joint. In rheumatoid arthritis, the inflammation leads to an increase in recruited immune cells to the synovial cavity. The recruited immune cells can then work to activate one another to further induce the inflammation present. The activated immune cells will secrete pro-inflammatory cytokines and enzymes that will contribute to joint by forming a pannus. The pannus is a tumor like tissue that allows immune cells to gain access to bone and cartilage, furthering the degradation of cartilage and bone, propagating chronic inflammation.

1.3.1.3 IL6-JAK-STAT signaling

The IL-6-JAK-STAT signaling pathway is critical in inflammatory conditions such as rheumatoid arthritis and has been widely studied for its role in driving chronic inflammation [65-68]. The IL-6 type cytokines exert their action through the activation of JAK-STAT pathway. The receptors involved in IL-6 signaling are the alpha receptor (IL-6R-α) and the transducing receptor gp130 (IL-6R-β) [69]. IL-6 can bind to membrane bound IL6-R and gp130 in cell types such as hepatocytes, neutrophils, monocytes, B cells, and T cells through classical signaling (Fig. 0.2a) [69, 70]. IL-6 can also bind to the soluble form of the receptor, sIL6-R. The IL6/sIL6-R complex can bind and activate gp130-expressing cells, known as *trans*-signalling (Fig. 0.2c) [71, 72]. The receptor gp130 is ubiquitously expressed in cells, allowing those cells not expressing transmembrane IL6-R to respond to IL-6 through *trans*-signaling [73]. Classic signaling is typically involved in a protective response, while *trans*-signaling induces pro-inflammatory responses [74]. In human serum, both sIL6-R and soluble gp130 are found and may act naturally to modulate systemic responses to the circulating IL-6 [69, 75-78]. However, in disease states, sIL6-R bound to IL6 that was produced by limited proteolysis can act to stimulate other immune cells, extending inflammation.

The initial signaling complex formation occurs in both classic and *trans*-signaling to induce the interaction of JAKs with the cytoplasmic portion of gp130 [79, 80]. Upon interaction, the JAK family members (JAK1, JAK2, and TYK2) become tyrosine phosphorylated and serve as binding sites for the transcription factor, signal transducer and activator of transcription (STAT3) [69, 81-83]. STAT3 is phosphorylated on a tyrosine residue (Y705), leading to the heterodimer formation of STAT3. Upon being phosphorylated in the cytosol, the dimerized STAT3 rapidly translocates to the nucleus where it binds to DNA to induce STAT-specific gene expression [84]. STAT3 also works to negatively regulate the JAK-STAT signalling by inducing the expression of cytokine signaling 3 (SOCS3) to prevent the excess level of inflammation [85, 86]. In addition to classical and *trans*-signaling, IL-6 can also activate gp130 intracellularly in the endosomal compartment when the IL-6 bound IL6-R is taken up by endocytosis (Fig. 0.2b) [87].

Many studies have found that STAT3 activation promotes inflammation and joint erosion in arthritis [88]. Specifically, in RA, the phosphorylation of STAT3 is increased in the synovium compared with healthy controls [89, 90]. The level of p-STAT3 (Y705) in resting T cells and monocytes from RA patients was found to be significantly higher than in healthy volunteers [68, 91]. IL-6 levels were also elevated in the plasma of RA patients and are correlated with the level of STAT3 phosphorylation in T cells and monocytes [68, 91]. The increased activation of p-STAT3 in RA may be effective as a potential biomarker for RA, alongside the increased expression of serum and synovial fluid IL-6 levels.

1.3.1.4 Pro-inflammatory mediators

Cytokines are proteins released by immune cells as a form of cell-to-cell communications to regulate cell division, differentiation, stimulation, and chemotaxis [92]. In inflammatory settings however, cytokines can potentiate the situation by further inducing and recruiting immune cells to the area [93].

In RA patients, the monocytes, macrophages, fibroblasts, and T cells recruited to the synovial cavity release pro-inflammatory mediators upon stimulation [94-96]. Common cytokines found in the synovial fluid of RA patients are IL-6, TNF-α, and IL-1β [97]. These cytokines can be amplified by the interaction between antigen presenting cells and CD4+ T cells [98]. Macrophage activation results in abundant production of pro-inflammatory cytokines that stimulate the cartilage's synovial fibroblasts and chondrocytes [98]. This can lead to the degradation of the proteoglycans and collagen in the tissue by secretion of enzymes [98]. Several studies have found inflammatory cytokines, including IL-17, IL-6, TNF-α, and IL-1β to be drivers of inflammation in RA patients [99, 100].

Many studies have found that IL-6 is critical in the pathogenesis of RA, as its levels are increased in the serum and synovial fluid of RA patients compared to healthy patients [101]. The IL-6 levels in the serum and synovial fluid levels correlate with the severity and activity of RA [101-103]. Further, IL-6 can induce the development of Th17 cells while preventing the differentiation of regulator T cells [104]. Th17 cells have a beneficial role in protecting against microbial infections but are also involved in many autoimmune conditions, like RA [104]. A

downstream cytokine of IL-6, MCP-1, which is responsible for the recruitment of monocytic phagocytes is also high in RA sera and synovial fluid when compared to healthy patients, indicating its importance in disease progression [105, 106]. Similarly, TNF-α levels in RA patients' serum and synovial fluid are significantly higher than in healthy individuals [107]. Due to the pro-inflammatory nature of these cytokines and their prevalence in both RA patients' sera and synovial fluid, they have the potential to be used as biomarkers for this inflammatory disease.

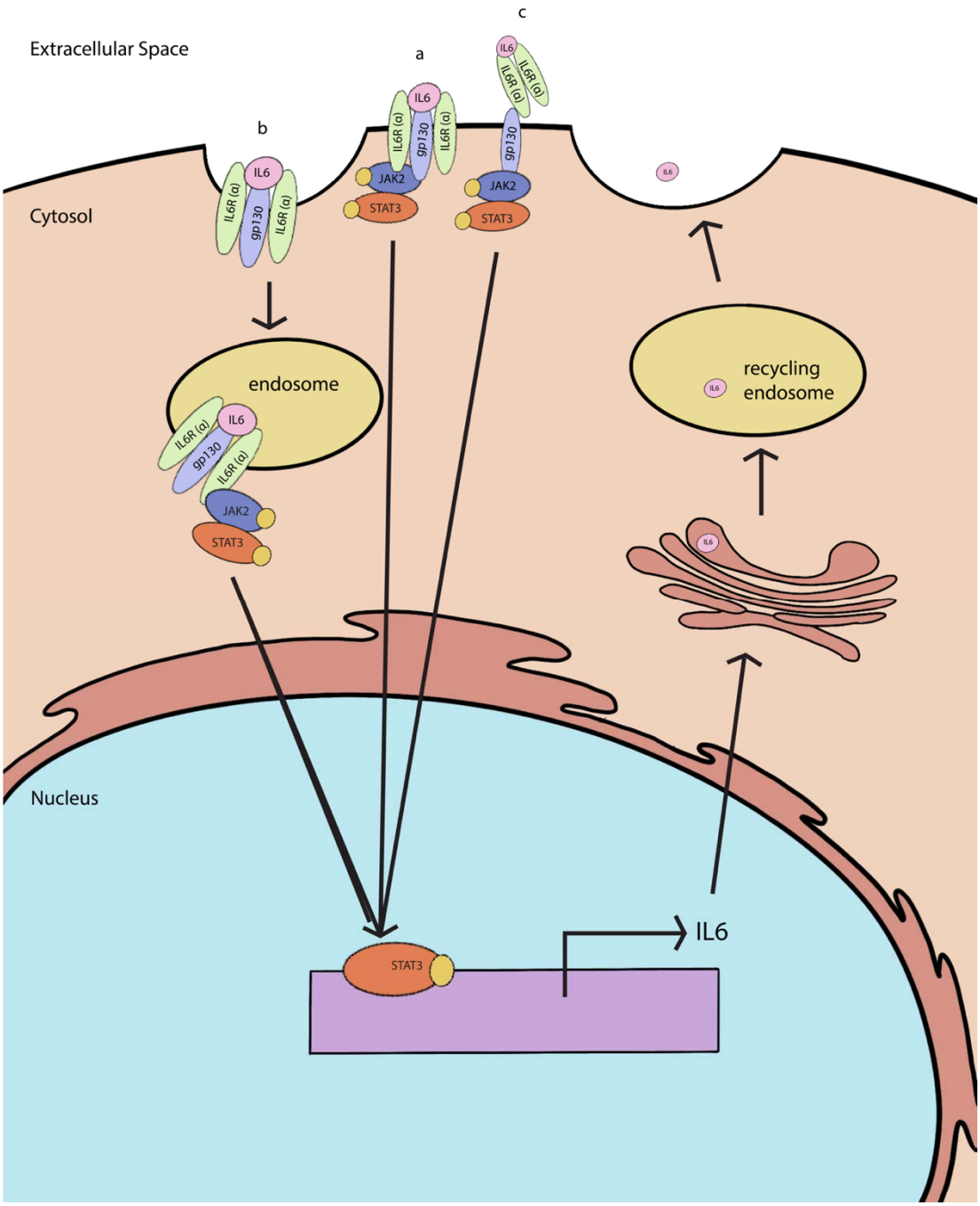

Figure 0.2. IL-6-JAK-STAT signaling. IL-6 can bind membrane bound gp130 and IL6-R to activate STAT3, known as classical signaling (**a**). IL-6 bound to soluble IL-6R can also bind to membrane bound gp130 to induce the activation of STAT3, which is known as *trans*-signaling (**c**). IL-6 can also activate gp130 through the endosome, after the uptake of the IL-6 bound IL-6R in endocytosis (**b**).

1.3.1.5 Current therapies for Rheumatoid Arthritis

Disease modifying anti-rheumatic drugs (DMARDs) are a class of drugs used for the treatment of inflammatory conditions such as rheumatoid arthritis, lupus, and inflammatory bowel disease [108]. Typically, they are divided into two classes, the conventional DMARDs and biological DMARDs [109]. The conventional DMARDs include methotrexate, leflunomide, hydroxychloroquine, and sulfasalazine [109]. Methotrexate is the most commonly used DMARD for RA, however its exact mechanism of action at a low dose in RA remains unknown. Methotrexate was originally used as an anti-cancer agent due to its low toxicity profile compared to other agents [110]. The most likely methotrexate effect at a low dose is its ability to increase the adenosine level and reduce pro-inflammatory cytokine levels [111, 112]. Through alteration of adenosine, methotrexate has an anti-inflammatory effect by decreasing the secretion of TNF, IFN-γ, IL-6, and phagocytosis [113]. The most common adverse effects are hepatotoxicity, ulcerative stomatitis and leukopenia [114]. Methotrexate may also cause an increased risk of infections, renal toxicity, neurotoxicity, and gastro-intestinal toxicity [115].

Leflunomide, is an anti-rheumatic drug used because of its ability to regulate the cell cycle progression by preventing *de novo* pyrimidine ribonucleotide biosynthesis [116]. Leflunomide is a pro-drug and is rapidly metabolized into an active metabolite form [116]. Its mechanism of action in rheumatoid arthritis is through its ability to regulate lymphocyte proliferation, inhibition of tyrosine kinase activity, and the inhibition of *de novo* pyrimidine synthesis [116]. While leflunomide does provide remission for some patients, its side effects are discouraging with diarrhea, alopecia, hepatic dysfunction, and hypertension occurring in many patients [117].

Sulfasalazine is another DMARD commonly used in the treatment of RA [118]. Sulfasalazine has a short latent period before its onset of action compared to other DMARDs [118]. However, the exact mechanism of how sulfasalazine or its metabolites work in providing efficacy in RA is still not clear [118]. *In vitro* assays have demonstrated that sulfasalazine and its metabolites can inhibit the release of pro-inflammatory cytokines such as IL-1, IL-2, IL-6, and IL-12 [118]. A study with animal models supported the immunomodulatory action of sulfasalazine and the down-regulation of pro-inflammatory cytokines [118]. Further, it has been

suggested that sulfasalazine also may work to inhibit RA progression by the inhibition of transcription factor nuclear factor kappa-B [119], inhibition of osteoclast formation [120], and induction of converting adenine nucleotides to adenosine [121]. The most common side effects of sulfasalazine are gastrointestinal problems, headache, and rash [118].

Hydroxychloroquine belongs to a class of drugs known as the 4-aminoquinolines and was originally used as an anti-malarial agent [122]. Hydroxychloroquine has a different mechanism than other DMARDs, as its original intent was not for inflammatory conditions [123]. The proposed mechanism of hydroxychloroquine for rheumatoid arthritis is through its ability to increase the lysosomal pH in antigen presenting cells [124]. This leads to a blockade of toll-like receptors on dendritic cells, preventing the action of the cells and reducing the inflammatory markers secreted by them [125, 126]. The increase in pH of acidic organelles also results in a blockade of the processing of antigenic peptides that can induce autoimmune response [124]. Hydroxychloroquine can accumulate in the acidic organelles because it is a weak base, which upon accepting protons within the lysosome becomes trapped, leading to a rise in pH [127, 128].

Hydroxychloroquine was initially created to replace chloroquine to reduce the incidences of retinopathy in patients [129]. However, the risk is not completely eliminated, and some patients still experience retinopathy symptoms [129]. Hydroxychloroquine must be administered for 3-6 months before effects are seen in RA patients, and often this time period is ineffective if the patient is undergoing a severe flare [130, 131]. The long period of time hydroxychloroquine requires to build up in the body is based on the pharmacokinetics and time the drug needs to fully saturate the lysosome [132, 133]. Common side effects of the drug are vomiting, muscle weakness, changes in vision, and headache.

Traditionally, if the use of a single DMARD leads to a disease flare or toxicity, the drug is substituted with an alternative DMARD [134]. However, many studies have shown that this strategy does not lead to long term remission [134]. In fact, sustained remission rarely occurs, as monotherapy with DMARDs tends to be ineffective [134]. Due to the lack of efficacy with monotherapy, combinational therapy has been used to increase the efficacy whiles maintaining a favourable side effect profile [135]. Typically, with the combinatorial approach, more aggressive

combinations of DMARDs occur if the patient continues to not respond to single agents [135]. In many cases, combinations of two or more DMARDs are used to attempt to attain a remissive state in patients. Triple therapy, for example is the combination of methotrexate, sulfasalazine, and hydroxychloroquine that has been used for the treatment of RA.

Biological DMARDs are typically used after the failure of conventional therapy or in cases of severe disease activity. Some examples of biological DMARDs are adalimumab, etanercept, rituximab, tocilizumab among others. In contrast to conventional therapies, biologics are highly specific and target specific cell type or pathways of the immune system [109]. Tocilizumab for example targets and binds to soluble and membrane-bound IL-6 receptors, blocking IL-6 signal transduction [136]. Tocilizumab is administered intravenously every four weeks and has shown effectiveness in moderate to severe RA as monotherapy and in combination with methotrexate [136]. In Canada, it is only recommended as a treatment course after a patient has failed to respond to one or more DMARD or tumour necrosis factor antagonist [137]. However, the adverse side effects of tocilizumab administration are infections, infusion reactions, gastrointestinal issues, and neutropenia [138].

Current slow-acting anti-rheumatic drugs are very limited in specificity and efficacy and have many side effects [139]. In addition, they also have limited effects on the long term prognosis of rheumatoid arthritis [139]. Despite new advancements in biological therapies targeting the signaling pathways involved in RA, few patients are able to achieve and maintain a state of drug-free remission. Therefore, the need for novel anti-inflammatories that are able to combat the chronic inflammation that occurs in RA, in a more specific manner are needed.

1.4 VR23

7-chloro-4-(4-(2,4-dinitrophenylsulfonyl)piperazin-1-yl)quinolone (VR23) is a quinoline-sulfonyl hybrid compound (Fig. 0.3), created in Dr. Hoyun Lee's lab in search of a novel anti-cancer agent. VR23 was derived using chloroquine as the parent molecule. Prior studies have documented its proteasome inhibition properties by targeting the β2 subunit of the 20S proteasome [140]. We have further analyzed VR23 in mouse models of cancer and found that VR23 was able to selectively kill cancer cells over non-cancer cells [140]. In addition, when

VR23 was administered in combination with other anti-cancer agents such as paclitaxel, it was able to reduce the toxic side effects [140].

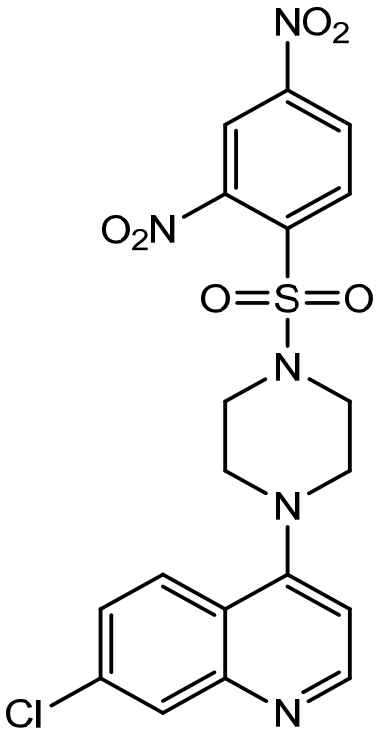

Figure 0.3. Chemical structure of VR23. [140, 141]

1.5 Objective

The parent molecule of VR23 is chloroquine, which has been modified to become hydroxychloroquine, which is used in the clinics as a DMARD for rheumatoid arthritis. Further, VR23's unique ability to reduce other anti-cancer drugs' side effects may show that it has potential as an anti-inflammatory agent. With this evidence, I hypothesized that VR23 had anti-inflammatory properties. To study this, initially, I investigated its ability to reduce acute inflammation through *in vitro* and *in vivo* experiments representing acute lung inflammation. Further, I analyzed whether VR23 can prevent chronic inflammation, and help patients diagnosed with autoimmune conditions like rheumatoid arthritis. Lastly, I investigated the mechanism by which VR23 works to reduce inflammation.

2.0 Chapter 1: The anti-tumor VR23 also shows strong anti-inflammatory effects on human rheumatoid arthritis cell models and acute lung inflammation in mice

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Abbreviations used in this article: ALI, acute lung injury; BALF, bronchoalveolar fluid; BTZ, bortezomib; CFZ, carfilzomib; CQ, chloroquine; DEX, dexamethasone; DMARD, disease modifying anti-rheumatic drug; ELISA, enzyme linked immunosorbent assay; HCQ, hydroxychloroquine; HFLS, human fibroblast-like synoviocyte; IL-1β, interleukin-1 beta; IL-6, interleukin-6; IL-8, interleukin-8; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; MPO, myeloperoxidase; NSAID, non-steroidal anti-inflammatory drug; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; TGF-β, tumor growth factor-beta; TNF-α, Tumor necrosis factor-alpha

Abstract

We previously found that the novel VR23 proteasome inhibitor not only possesses an effective anti-tumor activity without causing any ill-effects to animals but also reduces side effects caused by a partner drug when used in combination. Here we report that VR23, unlike other proteasome inhibitors, exhibits potent anti-inflammatory activity. In the lipopolysaccharide (LPS)-induced THP-1 monocyte model, VR23 down-regulates pro-inflammatory cytokines IL-1β, TNF-α, IL-6, and IL-8 at a similar efficacy to dexamethasone. In contrast, two well-known proteasome inhibitors, bortezomib and carfilzomib do not effectively down-regulate these pro-inflammatory cytokines. Data from a study with SW982 synovial cell line and primary human synoviocytes showed that VR23 not only effectively down-regulates IL-6 but also inhibits cell migration. Interestingly, the IL-6 down-regulation by VR23 was significantly more pronounced in the primary synovial cells from rheumatoid arthritis (RA) patients than those from healthy donors, suggesting that VR23 can be selective against RA. Finally, VR23 effectively reduces neutrophil migration, TNF-α secretion, and tissue inflammation in mice (female Balb/c strain) with an LPS-induced acute lung injury. Thus, our current data indicate that VR23 can be effective on both acute and chronic inflammatory conditions. Taken together with our previous work, VR23 is not only effective on inflammatory conditions but also applicable to different aspects of cancer control including the treatment and prevention of tumor development by chronic inflammatory responses.

Introduction

Well-regulated inflammatory response is essential for the control of microbial infections and clearing dead cells and other debris. However, its dysregulation may lead to general inflammatory conditions such as acute lung inflammation (ALI), pneumonia, and rheumatoid arthritis (RA). It is also increasingly clear that chronic inflammation often results in permanent genomic alterations, eventually leading to the initiation, promotion and malignant conversion of tumors [142-145]. Many different inflammatory mediators including pro-inflammatory cytokines, growth factors and free radicals are involved in these processes [142, 145]

Currently, non-steroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids are most often used to treat general inflammatory conditions [146]. Unfortunately, however, these drugs often cause toxic gastro-intestinal side effects such as ulceration and hemorrhage [146]. In

addition, NSAID also increases the risk of vascular and coronary problems, heart failure, and hypertension [147, 148]. Glucocorticoids are a class of corticosteroids that up-regulate anti-inflammatory proteins and down regulate multiple inflammatory genes [149]. Although dexamethasone (DEX), one of the most widely used glucocorticoids, is very effective in reducing inflammation, it can also lead to serious toxic side effects if used over a prolonged period [150, 151]. The common side effects include musculoskeletal weakness, endocrine abnormality, pancreatitis, increase of blood pressure, and problems in cardiovascular and central nervous systems, underscoring the importance of developing effective new drugs with minimal side effects [152].

Autoimmune diseases are difficult to control and, unfortunately, the incident rates are on the rise [29, 153]. One of the most common autoimmune diseases is RA, which results in inflammatory arthritis of the joints and, in some cases, bone and cartilage destruction [154]. Like in many other autoimmune conditions, a high level of pro-inflammatory cytokines is usually involved in the initiation and progress of RA [29]. Rheumatoid arthritis is currently treated with disease modifying anti-rheumatic drugs (DMARDs) such as methotrexate, hydroxychloroquine (HCQ), glucocorticoids, and biological agents [155]. However, monotherapy with these agents typically is ineffective, due to the development of drug resistance [156]. The combination of conventional DMARDs with biological agents is effective for 50% of RA patients, allowing them to achieve clinical remission [156]. However, for the remaining 50% of patients, there is insufficient disease reduction [156]. Therefore, RA still requires the development of new drugs with novel mods of actions.

We previously reported the synthesis and characterization of 7-chloro-4-(4-(2,4-dinitrophenylsulfonyl) piperazin-1-yl) quinolone 7-chloro (VR23) , which possesses effective anti-tumor activity [140, 157]. The major molecular target of VR23 is β2 of the 20S proteasome catalytic subunit [158]. It was suggested previously that proteasome inhibitors may possess anti-inflammatory property, since they can theoretically down-regulate the NF-κB pathway by inhibiting Ikβ degradation [159-161]. However, this assumption remains controversial as others have shown pro-inflammatory activity of certain proteasome inhibitors [162, 163].

Chloroquine (CQ) and hydroxychloroquine (HCQ) possess anti-inflammatory properties [164]. In addition, CQ/HCQ can decrease reactivity against auto-antigenic peptides by interfering antigen processing, thus effectively controlling autoimmune disorders such as RA and

systemic lupus erythematosus (SLE) [124, 165]. As a quinoline-sulfonyl hybrid compound, VR23 contains the main scaffold of CQ. Therefore, we hypothesized that VR23 may also possess anti-inflammatory and anti-autoimmune activities. We here report that VR23 does indeed possess these properties – just much stronger than CQ/HCQ.

Materials and Methods

Reagents

VR23 was synthesized by Dalton Pharma Services (Toronto, ON, Canada) following the protocol described previously [157], and its stock solution (20 mM) was prepared in dimethyl sulfoxide (DMSO) as previously described [166]. Bortezomib (BTZ), Carfilzomib (CFZ), Cell Counting Kit-8 (CCK-8) (Enzo Life Sciences) were purchased through Cedarlane Inc. (Burlington, ON, Canada). The following items were purchased from Sigma Aldrich (Oakville, ON, Canada): DEX (>97%), HCQ sulfate (>98%), lipopolysaccharide (LPS) from *Escherichia Coli* O111:B4. The following items were purchased from Thermofisher Scientific (Waltham, MA): tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), RPMI 1640, high glucose Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS) and antibiotic and antimycotic solutions. The single-analyte enzyme linked immunosorbent assay (ELISA) kits were purchased from Qiagen (Montreal, QC, Canada).

Cell lines and cell culture

THP-1 and SW982 cells, purchased from the American Type Culture Collection (ATCC), were cultured in RPMI-1640 (the former) or high glucose DMEM (the latter) supplemented with 100 μg/mL streptomycin, 100 units/mL penicillin and 10% (v/v) FBS. The THP-1 and SW-982 cell lines were used at passages less than 10. Primary human fibroblast-like synoviocytes from three healthy donors (HFLS-N; catalog number 408-05a) and three donors with rheumatoid arthritis (HFLS-RA; catalog number 408RA-05a) were purchased from Cell Applications Inc. (San Diego, CA) through Cedarlane Inc. (Burlington, ON, Canada). The cells were cultured in synoviocyte growth medium from the same supplier. The cells in each vial were isolated from a single donor and provided at passage 1, and all experiments were conducted at passage 3-8, as described previously [167].

Determining IC_{50} values using the sulforhodamine B (SRB) colorimetric assay

The SRB assay [168] was used to determine the drug concentration that half of the cell growth/proliferation is inhibited (IC_{50}). Cells were cultured in 96-well plates, with 5,000 cells in 100 μL of culture medium per well. The rest of the protocol was described previously [169, 170].

Cell Counting Kit-8 assay

Cells were cultured in 11 columns of 96-well plates, with approximately 5,000 cells in 100 µL of culture medium per well, excluding one column for a medium-only control. The plates were incubated overnight in a humidified atmosphere at 37°C with 5% CO_2 and 95% air. Following incubation, the plates were treated with test compounds diluted in media at a total volume of 100 µL. Each column was treated with a different condition, and each treatment consisted of 8 repeats to ensure accuracy of the results. The plates were then incubated for 6 h in a cell-culture incubator, and then added 10 µL of CCK-8 reagent to each well, followed by the incubation of additional 2 h to allow colour formation. The absorbance was measured using an automatic plate reader (Synergy H4 Hybrid Multi-Mode Microplate Reader, BioTek) at 450 nm wavelength. Finally, cell viability was determined using the following equation: cell viability (%) = (Abs_{trtmt}- Abs_{media})/(Abs_{UT}- Abs_{media}) × 100, where Abs_{trtmt} is the absorbance value of cells with the various treatment conditions; Abs_{media} is the absorbance of the wells containing media only; Abs_{UT} is the absorbance of the wells with untreated cells.

Enzyme-linked immunosorbent assay (ELISA)

THP-1 cells were plated at the density of 500,000 cells/mL in 1 mL volume, and the SW982 and HFLS cells were at 50,000 cells/mL in 0.5mL for cytokine analysis. Single Analyte ELISArray human kits for each of the individual cytokines, tumor necrosis factor (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and interleukin-8 (IL-8) (all from Qiagen) were used following recommendation by the supplier. A mouse TNF-α ELISA Kit (Thermofisher) was used for the analysis of cytokine secretion in the mouse bronchoalveolar lung fluid.

Cell migration assay

Cell migration assays were performed using Corning Invasion chambers with matrigel matrix (Fisher Scientific). The lower chamber contained compounds (e.g., VR23 or HCQ) and chemo-attractant (e.g., 10% FBS). The upper chamber was loaded with 25,000 cells in FBS-free medium. The analysis of the trans-well inserts was performed as described previously [171].

After 24-h incubation, the trans-well insert was removed, and then the remaining culture medium and cells on the upper chamber were also removed from the top of the membrane with cotton swab. The trans-well insert was placed back into the well containing 1 mL of 70% ethanol for 10 min to fix the cells. The residual ethanol was carefully removed with cotton swab. Cells on the trans-well insert membrane were air-dried for 15 min at room temperature. To stain cells, the trans-well insert containing cells was placed in 1 mL of 0.2 % (w/v) crystal violet for 10 min. The excessive crystal violet was carefully removed from the membrane with cotton swab. Cells on the membrane were air-dried at room temperature prior to analysis by the Olympus IX73 inverted microscope. Cell images (10×) were captured at five different fields. The average number of cells that migrated through the membrane towards the chemo-attractant was determined. Adherent cells attach to the membrane on the lower chamber side upon migration, and suspension cells drop into the lower chamber.

Animal work

Female Balb/c mice strain 028 (6-8 weeks old), purchased from Charles River Laboratories (Senneville, QC, Canada), were fed regularly with laboratory chow and water upon arrival at the Laurentian University Animal Care Facility (Sudbury, ON, Canada) where this work was carried out.

To determine an optimal dose of LPS, three mice per group were treated intranasally for 24 h under the following three different conditions: (i) no LPS, (ii) 0.25 mg of LPS per kg of body weight, and (iii) 0.50 mg of LPS per kg of body weight. After the animals were sacrificed, the bronchoalveolar lavage fluid (BALF) of each mouse was collected and analyzed for LPS-induced inflammatory response. The results indicated that 0.40 mg/kg (i.e.; between 0.25 mg/kg and 0.5 mg/kg) is the optimal concentration.

Experimental design involving the use of VR23 and the control was based on acute lung injury instillation described by Wei and Huang [172]. Briefly, mice were divided into 5 groups, with 3-5 mice in each as follows: no acute lung injury (i.e., no treatment), sham treatment, LPS only, LPS + DEX, and LPS + VR23. VR23 (30 mg/kg) and DEX (4 mg/kg) were intraperitoneally (i.p.) injected 1 h prior to LPS administration. The mice were given LPS (0.4 mg/kg) in phosphate buffered saline (PBS) intranasally to induce acute lung injury. The sham treated mice were given PBS plus DMSO (i.p.; since VR23 was dissolved in DMSO). The mice

in the no-acute-lung-injury group were not given any drugs or stimulation. The mice were sacrificed by isoflurane inhalation at 24 h post-induction of ALI with LPS. Bronchoalveolar lavage fluid was collected and cells infiltrated into the bronchoalveolar fluid were counted. The fluid was also used to measure the amount of TNF-α by ELISA. The lungs were removed to determine the levels of myeloperoxidase (MPO) and histopathological analysis. The animal study protocol was reviewed and approved by the Animal Care Committee at Laurentian University.

Myeloperoxidase (MPO) assay

The accumulation of neutrophils in the lung tissue was analyzed using a colorimetric MPO assay kit (Sigma Aldrich), which measures the amount of myeloperoxidase activity. After 24 h of lung injury induction, the upper and lower lobes of the right lung were snap-frozen and kept at -80°C until analysis. Immediately prior to experiments, the lungs were weighed, thawed, and homogenized using cold MPO assay buffer (included in the kit) with a sonic dismembrator. The MPO activity was then determined as per the protocol provided by the supplier (Sigma Aldrich).

Histopathological analysis

Following 24 h of the lung injury induced with LPS, lung tissues were embedded in paraffin blocks, followed by slicing them into 10 μm sections using a microtome. Each slice was stained with haematoxylin and eosin (H & E) for examination of cell morphology and tissue type. For microscopy, at least 10 fields were analyzed per sample using an Olympus IX73 microscope (20× magnification).

Statistical analyses

Each experiment was repeated in three biological replicates unless otherwise indicated. The mean values of these results were used for statistical analysis and expressed as mean ± standard error. Comparison between experimental groups was made by *p* value determination using one-way ANOVA. The *p* value of <0.05 is considered to be statistically significant. The Dunnett's post-hoc test was performed when necessary, to determine the significance between the treatment groups and controls. Analyses were performed using GraphPad Prism software, version 7.0e (San Diego, CA).

Results

VR23 effectively down-regulates the level of pro-inflammatory cytokines in monocytes

To determine the optimal concentrations of LPS and TNF-α in stimulating monocyte cells, we measured the level of tumor growth factor-β (TGF-β) or IL-8 induction in the presence of various concentrations of LPS or TNFα (Figs. 1.5 and 1.6). The resultant data determined by ELISA indicated that the optimal doses of LPS and TNF-α are 5 μg/mL and 10 ng/mL, respectively. Thus, throughout this work, we used these concentrations unless stated otherwise. Similarly, we determined optimal time post-stimulation. As shown in Fig. 1.5c-g, the levels of secreted cytokines IL-1β, TNF-α and IL-8 reached a plateau at 6 h after stimulation. One exception was the level of IL-6 induced by LPS, which never reached a peak by 24 h post-stimulation, the last time point used in this experiment. Nevertheless, the 6 h post-stimulation time point could still be used to measure the relative amounts of secreted IL-6.

As VR23 contains the main scaffold of the anti-inflammatory CQ, we examined whether VR23 can also down-regulate inflammation. To standardize the doses for different compounds used for comparison, we first determined the values of growth inhibition by 50% (IC_{50}) for SW982 and THP-1 cells in response to VR23, CQ, HCQ, BTZ, CFZ or DEX. We then determined changes in the levels of four major pro-inflammatory cytokines after THP-1 cells were stimulated with either LPS or TNF-α for 6 h. As shown in Fig. 1.1a and 1.1b, VR23 at 3 μM down-regulates IL-1β and IL-6 secretion to near the background level. At the same concentration, VR23 also substantially (approximately 50%) down-regulates the level of TNF-α (Fig. 1.1c). VR23 down-regulates IL-8 by 19% and 76% at 3 μM and 6 μM, respectively, in LPS-stimulated THP-cells (Fig. 1.1e). Interestingly, under our experimental conditions, VR23 more effective down-regulates IL-8 in TNF-α stimulated THP-1 cells (60% at 3 μM and almost completely at 6 μM) (Fig. 1.1e).

In contrast, the other two proteasome inhibitors, BTZ and CFZ, do not down-regulate the levels of IL-1β, IL-6, TNF-α, or IL-8 at indicated concentrations. Thus, proteasome inhibitors do not necessarily have the same effects on the regulation of pro-inflammatory cytokines. Compared to VR23, the "parental" CQ is much less effective on the level of IL-1β and TNF-α at 25 μM, although it does down-regulate IL-6 in a similar level rendered by 3 μM VR23, indicating that VR23 is clearly superior to CQ in modulating pro-inflammatory cytokines. At the

IC_{50} concentration, DEX also effectively down-regulates IL-1β, TNF-α, and IL-6. However, VR23 is generally more effective than DEX (Fig. 1.1).

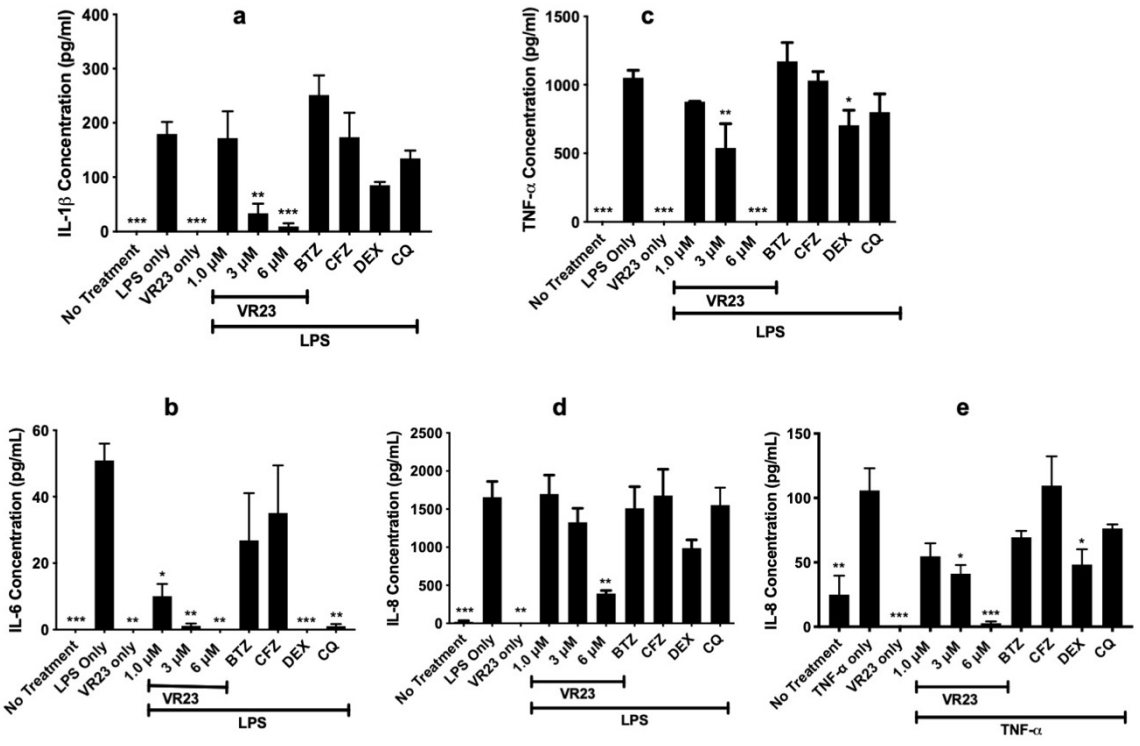

Figure 1.1. VR23 down-regulates pro-inflammatory cytokines. Shown are the levels of IL-1β (**a**), IL-6 (**b**), TNF-α (**c**) and IL-8 (**d**) in the supernatant of THP-1 cells stimulated with LPS (5 μg/mL) for 6 h in the absence (control) or presence of varying concentrations of VR23. (**e**) Shown are the levels of IL-8 after THP-1 cells were stimulated for 6 h with TNF-α (10 ng/mL) alone or in combination with varying concentrations of VR23. For comparison, bortezomib (BTZ, 6 nM), carfilzomib (CFZ, 1 nM), dexamethasone (DEX, 2 μM), and chloroquine (CQ, 25 μM) were also included. The comparison between groups was made using a one-way ANOVA. *, **, and *** are $p<0.05$, $p<0.01$, and $p<0.001$, respectively, which denote significant differences from the LPS only or TNF-α only group. *P* values were determined by a Dunnett's test, and the values presented are mean ± SEM (n=3).

VR23 effectively down-regulates IL-6 in human synovial cells from rheumatoid arthritis patients.

Having established that VR23 has strong anti-inflammatory activity in monocytes, the next question was whether VR23 can also regulate chronic inflammation, a major factor involved in the initiation and progress of autoimmune disease such as in RA patients. To address this question, a study was carried out with SW982, a cell line derived from human synovial sarcoma. The SW982 cell line is widely used as a model for studying rheumatoid arthritis, as it possesses similar immunological properties as primary synovial cells [173]. Since IL-6 is strongly implicated to the progress of RA [174], we measured IL-6 as its surrogate. We found that VR23 effectively down-regulates IL-6 by 6 h post-treatment in SW982 cells. HCQ also shows a trend of down-regulation, but with no clear significance (Fig. 1.2a). Note that HCQ was included in this experiment as it is often used to treat RA and SLE [128, 175, 176]. In addition, both HCQ and VR23 contain a 7-chloro-4-aminoquinoline group found in many anti-inflammatory molecules [177]. Our findings thus indicate that VR23 can be effective and potentially safe for the treatment of RA patients.

We next examined the effects of VR23 on primary human synovial cells. Our model in this study was human fibroblast-like synoviocytes isolated from the synovial fluid in joint capsules of healthy donors (HFLS-N) or RA patients (HFLS-RA). It is known that synoviocytes in RA patients produce harmful molecules that contribute to joint destruction and the exacerbation of the disease. For these experiments, we first determined the dose of compounds that are effective without killing cells at 6 h post-stimulation (Fig. 1.6). For both HFLS-N and HFLS-RA, up to 10 μM of VR23 did not affect cell viability either used alone or in combination with IL-1β (Fig. 1.6d-e). We then determined changes in the level of the IL-6 pro-inflammatory cytokine after HFLS-N or HFLS-RA cells were stimulated with IL-1β or LPS for 6 h. We found that both VR23 (5 μM) and HCQ (15 μM) down-regulate IL-6 by ~30% in comparison to the IL-1β stimulated sample in the HFLS-N cells (Fig. 1.2b). The picture is quite different in HFLS-RA cells: while VR23 (5 μM) down-regulated IL-6 by 63% in comparison to the IL-1β stimulated sample, while HCQ (30 μM) did so only by 45% (Fig. 1.2c). These data thus suggest that VR23 can be more effective in modulating IL-6 production on RA patients than healthy persons. When LPS is a stimulant, 2.5-5.0 μM VR23 effectively down-regulate IL-6 to the background level, while HCQ was ineffective (Fig. 1.2d,e). Therefore, HCQ is much less effective in down-

regulating IL-6 than VR23 on HFLS-RA cells stimulated by either IL-1β or LPS. These findings are consistent with data from the study carried out with SW982 (Fig. 1.2a), and strengthen the idea that VR23 could be an effective drug against RA.

The migration of human fibroblast-like synovial cells plays a key role in RA progression as they eventually invade and destruct the cartilage and bone [178, 179]. Since we previously found that VR23 prevents tumor cell spread to adjacent tissues [166], we examined whether VR23 also inhibits synovial cell migration. As shown in Fig. 1.3a and b, 5 μM of VR23 down-regulates cell migration to the background level when 10% FBS was used as chemo-attractant. We also examined the effect of VR23 on the level of monocyte chemoattractant protein-1 (MCP-1), secreted in both HFLS-N and HFLS-RA cells. As shown in Fig. 1.3c and d, VR23 at 5 μM down-regulates MCP-1 secretion to the background level in both HFLS-N and HFLS-RA. In contrast, HCQ is ineffective on both, HFLS-N and HFLS-RA. We also carried out a scratch-wound healing assay using an Incucyte S3 Live cell analysis system (Fig. 1.7). The scratch wound inflicted to the SW982 cell population was completely healed by 24 h post-scratch in the presence of 25 μM CQ. In contrast, even by 48 h post-scratch, the wound was not completely healed in the presence of 5 μM of VR23. Thus, these data indicate that VR23 is much better in preventing synoviocyte migration than CQ/HCQ. Together, our data again indicate that VR23 may be an effective treatment option against RA and possibly other autoimmune conditions.

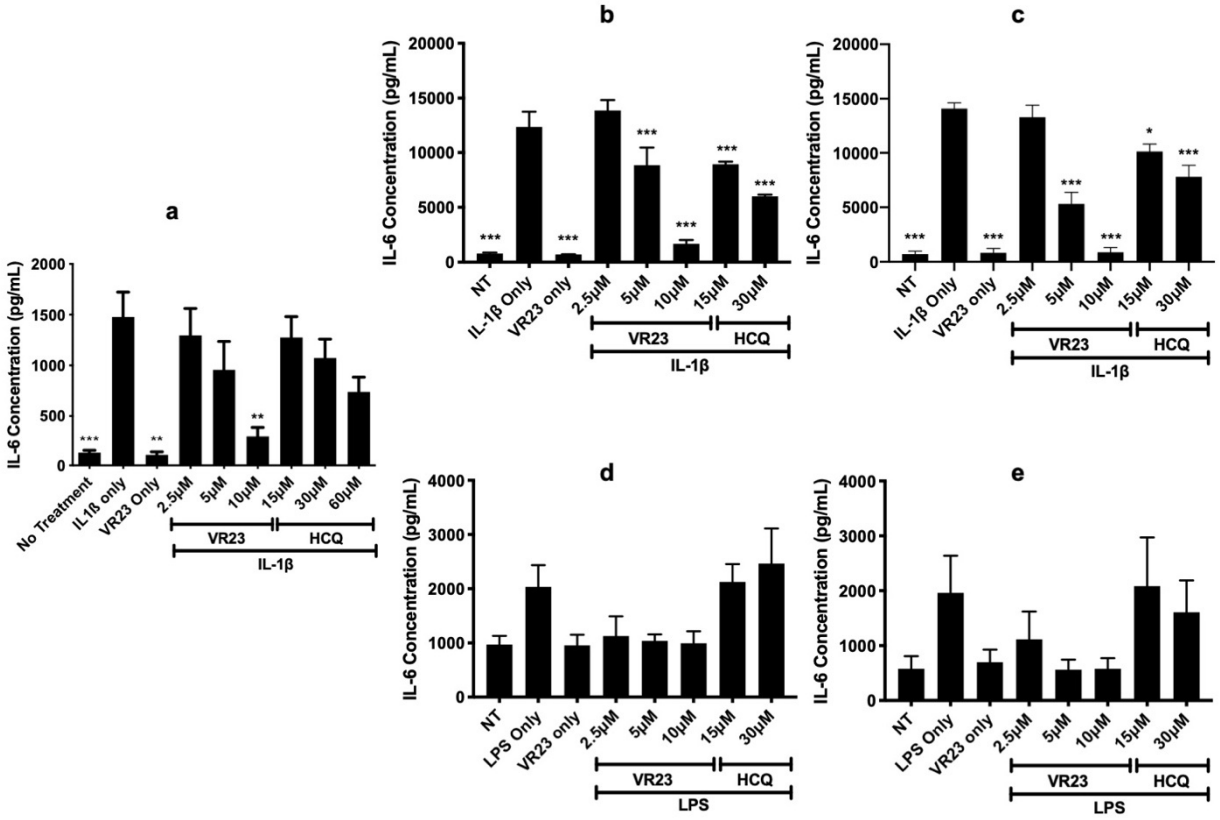

Figure 1.2. VR23 down-regulates IL-6 in human synovial cells. (**a**) The levels of IL-6 secreted in the supernatant of SW982 cells stimulated with IL-1β (1 ng/mL) for 6 h in the absence or presence of varying doses of VR23 or hydroxychloroquine (HCQ). (**b, c**) The levels of IL-6 in the supernatant of HFLS-N (**b**) or HFLS-RA (**c**) cells are shown at 6 h post-stimulation with IL-1β in the absence or presence of varying doses of either VR23 or HCQ. For "VR23 Only" samples, 5 µM VR23 was used. The values presented are mean ± SEM (n=3). (**d, e**) The levels of IL-6 in the supernatant of HFLS-N (**d**) and HFLS-RA (**e**) cells are shown at 6 h post-treatment with LPS alone (100 ng/mL), HCQ, or in the presence of varying concentrations of VR23. The values presented are mean ± SEM (n=3).The comparison between groups was made using a one-way ANOVA. *, **, and *** are $p<0.05$, $p<0.01$, and $p<0.001$, respectively, which denote significant differences from the IL-1β only or LPS only group, determined by a Dunnett's test. "n=3" shown above denotes three independent experiments with samples from three different individuals.

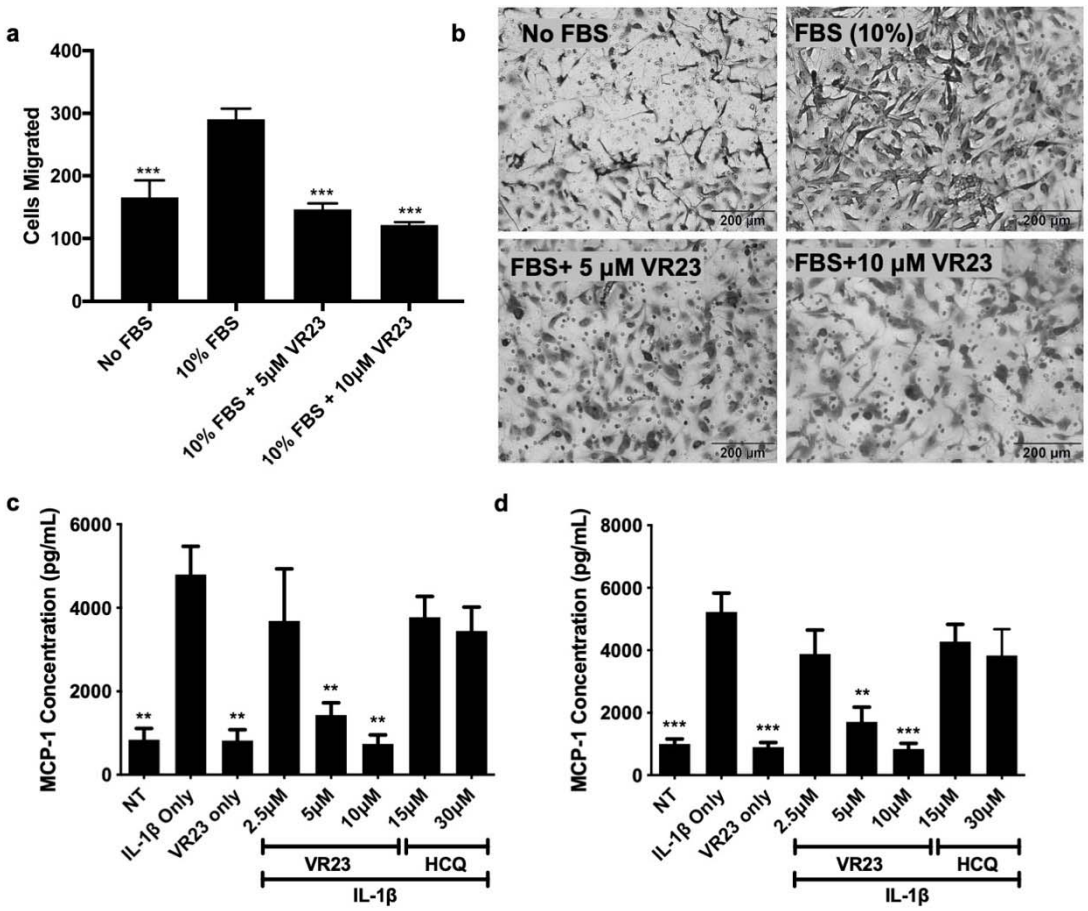

Figure 1.3. VR23 prevents synovial cell migration. (**a**, **b**) VR23 at 5 μM completely prevents HFLS-RA cell migration promoted by 10% FBS. An example of actual data is shown in **b**. Detailed protocol of the Matrigel matrix-based assay is described in the Materials and Methods. No FBS and FBS (10%) denote 0% FBS and 10% FBS included in the lower chamber, respectively. As described in panels, 10% FBS + 5 μM VR23 or 10% FBS + 10 μM VR23 were included in the lower chamber. An Olympus IX73 microscope (10× magnitude) was used to capture 10 separate fields for each sample. The values presented are mean ± SEM (n=2). Data in panels **c** and **d** show the levels of MCP-1 in the supernatant of HFLS-RA (**c**) and HFLS-N (**d**) cells treated with IL-1β (1 ng/mL) for 6 h in the absence (control) or presence of varying concentrations of either VR23 or HCQ. The values presented are mean ± SEM (n=3). The comparison between groups was made using one-way ANOVA. *, **, and *** are $p<0.05$, $p<0.01$, and $p<0.001$, respectively, which denote significant differences from the 10% FBS or IL-1β group, determined by a Dunnett's test.

VR23 is capable of treating acute lung injury in mice

Since our data from *in vitro* studies showed that VR23 can substantially reduce or almost completely prevent the secretion of pro-inflammatory cytokines in THP-1 and SW982 cells, we examined its anti-inflammatory effects using a mouse model. Toward this goal, we first determined an optimal LPS dose to induce TNF-α in the mouse bronchoalveolar. We found that 0.4 mg LPS per kg body weight is an optimal dose when the subjected animals' lungs were analyzed at 24 h post-LPS (Fig. 1.8).

Acute lung injury (ALI) is characterized by increased permeability of the capillary barrier, which is accompanied by an increase in lung edema and an influx of neutrophils into the bronchoalveolar space [180]. The influx of neutrophils caused by ALI induces the release of pro-inflammatory cytokines such a IL-1β, TNF-α, and IL-8 [180]. To determine the anti-inflammatory effects of VR23, the level of TNF-α in the BALF was measured at 24 h after the animal was treated with 0.4 mg of LPS. We found that VR23 effectively reduces the level of TNF-α (Fig. 1.4a). Furthermore, VR23 at 30 mg/kg body weight completely inhibited cell invasion into the bronchoalveolar space (Fig. 1.4b). In both cases, VR23 and DEX showed similar efficacy.

Myeloperoxidase is a marker distinctly correlated with inflammation. Neutrophils that influx into the bronchoalveolar space due to ALI caused by acute inflammation will degranulate and release the pro-inflammatory enzyme MPO [181]. Therefore, the level of MPO can be an excellent inflammation marker in lung [181]. Data in Fig. 1.4c show that VR23 is a strong inhibitor of MPO.

LPS-induced ALI can lead to lung tissue inflammation due to the influx of immune cells into the alveolar space. To gain insight into this aspect, we examined the effects of VR23 on lung tissue sections stained with H & E. The resultant histopathological data show that alveolar inflammation caused by LPS is largely prevented when animals are treated with 30 mg/kg VR23 (Fig. 1.4d). DEX treatment also showed a similar result (Fig. 1.4d). This suggests that the anti-inflammatory activities of VR23 observed in the cell culture system can be translated into a pre-clinical model.

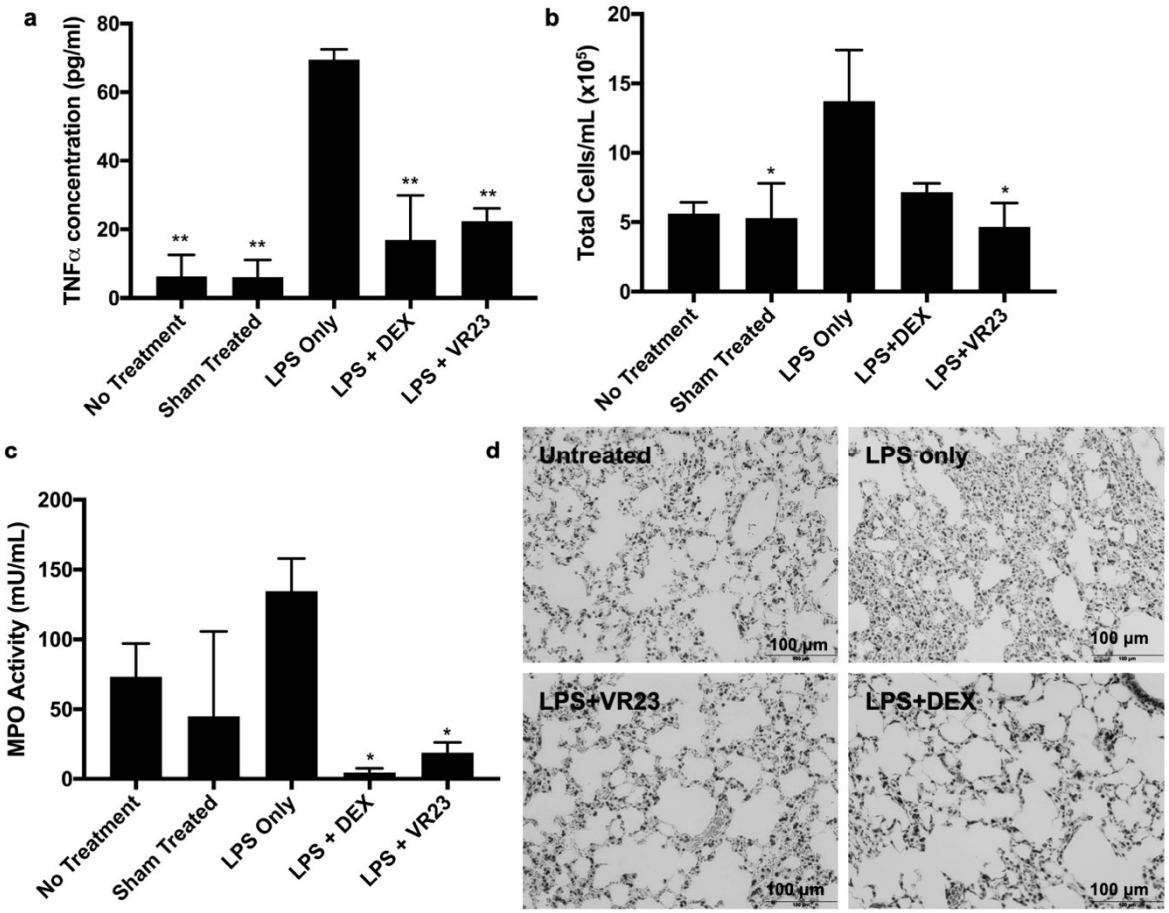

Figure 1.4. VR23 down-regulates the acute lung inflammatory response caused by LPS. (**a**) The level of TNF-α was measured by ELISA with bronchoalveolar fluid of mice with ALI induced with LPS (0.4 mg/kg). Mice with ALI were treated with 30 mg/kg VR23 at 1 h prior to the induction with LPS for 24 h. (**b**) The effects of VR23 on the total cell counts per mL of the bronchoalveolar fluid of mice with LPS-induced ALI. (**c**) The effects of VR23 on MPO production in the lung tissues of mice with LPS-induced ALI. **(d**) Histopathological images of LPS-induced acute lung injury. An Olympus IX73 microscope (20× magnitude) was used to capture 10 separate fields for each sample. The comparison between groups was made using a one-way ANOVA. *, **, and *** are $p<0.05$, $p<0.01$, and $p<0.001$, respectively, which denote significant differences from the LPS only group, determined by a Dunnett's test. The values presented are mean ± SEM (n=3-6).

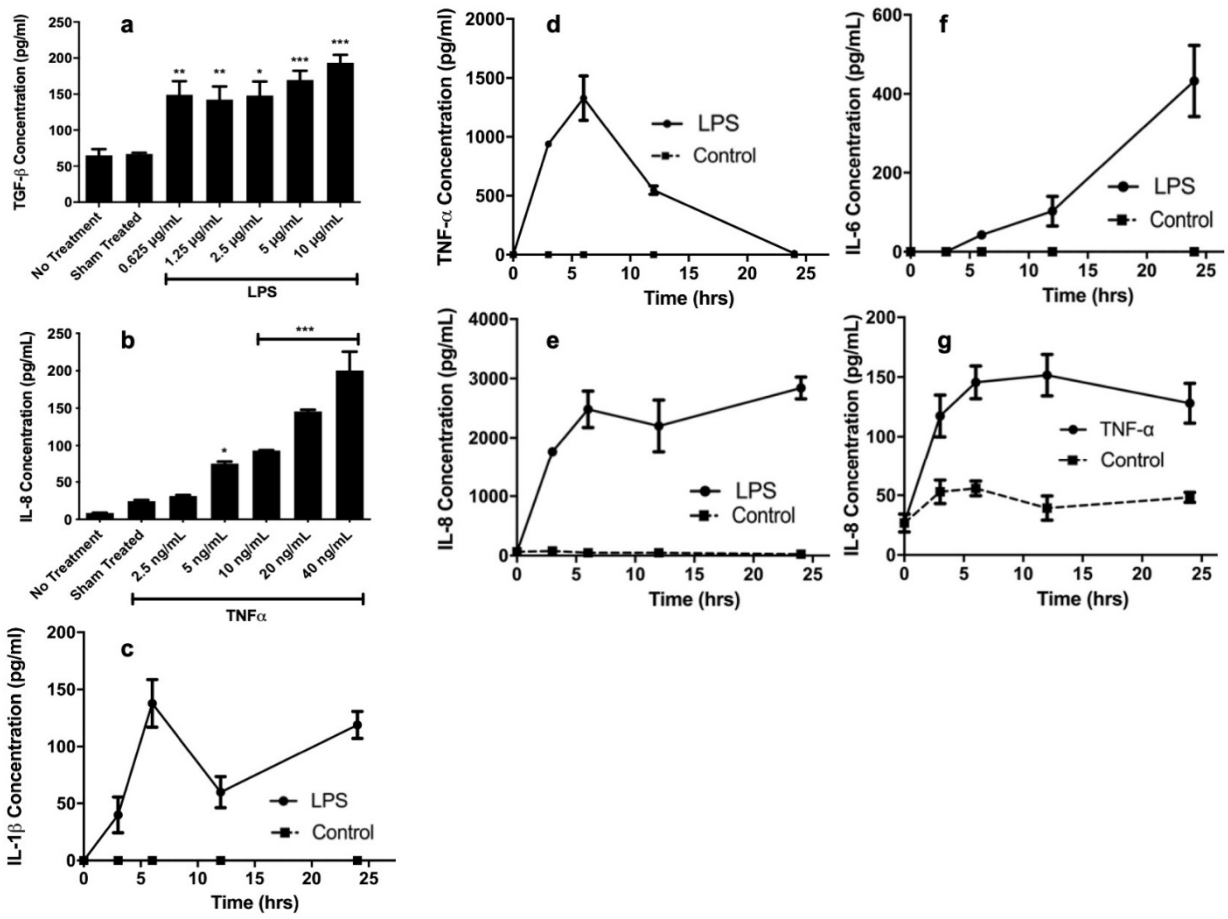

Figure 1.5. The levels of inflammatory cytokines secreted in the presence of LPS or TNF-α. (**a**) Levels of TGF-β secreted in the supernatant of THP-1 cells stimulated with varying concentrations of LPS. (**b)** Levels of IL-8 in the supernatant of THP-1 cells stimulated with varying concentrations of TNF-α. (**c**-**f**) Levels of IL-1β (**c**), TNF-α (**d**), IL-6 (**e**) and IL-8 (**f**) measured in the supernatant of THP-1 cells stimulated with LPS (5 µg/mL) as the function of time. (**g**) Levels of IL-8 in the supernatant of THP-1 cells stimulated with TNF-α (10 ng/mL) for different durations. The comparison between groups was made using one-way ANOVA. *, **, and *** are $p<0.05$, $p<0.01$, and $p<0.001$, respectively, which denote significant differences from the no treatment group, determined by a Dunnett's test. The values presented are mean ± SEM (n=3).

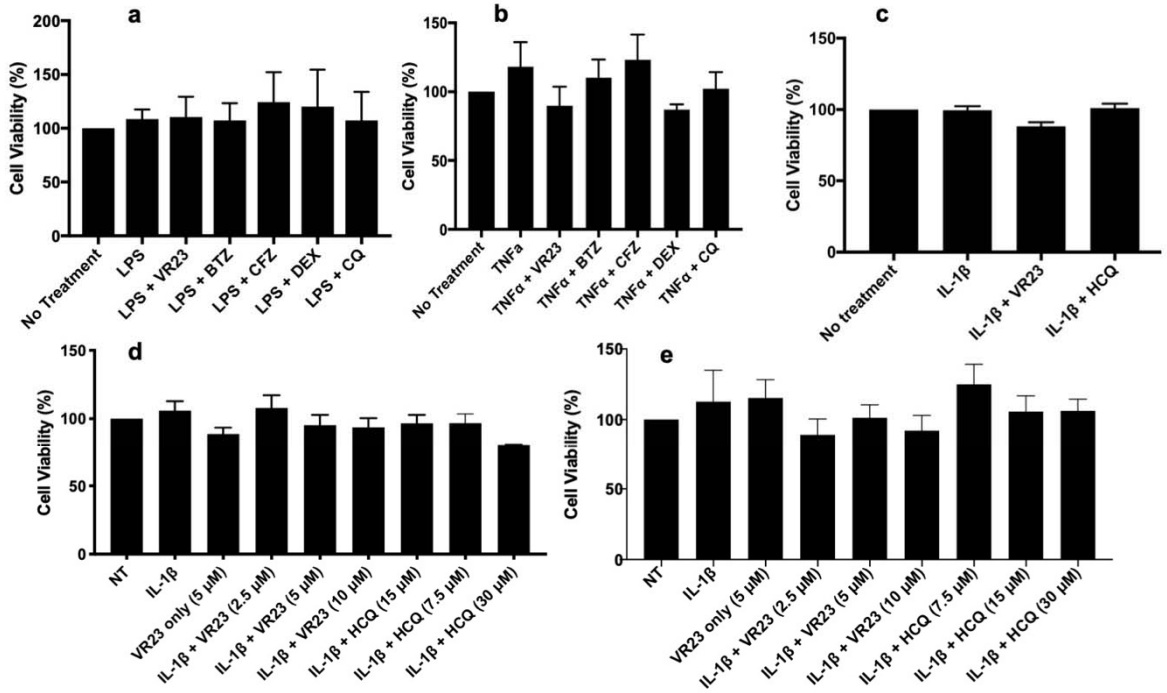

Figure 1.6. Cell growth and viability are largely unaffected under the experimental conditions employed in this study. (**a**-**d**) The viability rates are shown for THP-1 (**a,b**) or SW982 (**c**) cells after they were treated with LPS (5 μg/mL), TNF-α (10 ng/mL), or IL-1β (1 ng/mL) in the absence or presence of different compounds for 6 h. Results of the viability tests with HFLS-N (**d**) and HFLS-RA (**e**) cells (5,000 cells/well) measured at 6-h post-stimulation in the absence (control) or presence of varying concentrations of VR23 or HCQ. For abbreviation, see text. Figures A-C treated with compounds at their relative IC_{50} values. The comparison between groups was made using one-way ANOVA. * is $p<0.05$, which denotes a significant difference from the LPS,TNF-α, or IL-1β only group, determined by a Dunnett's test. The values presented are mean ± SEM (n=3).

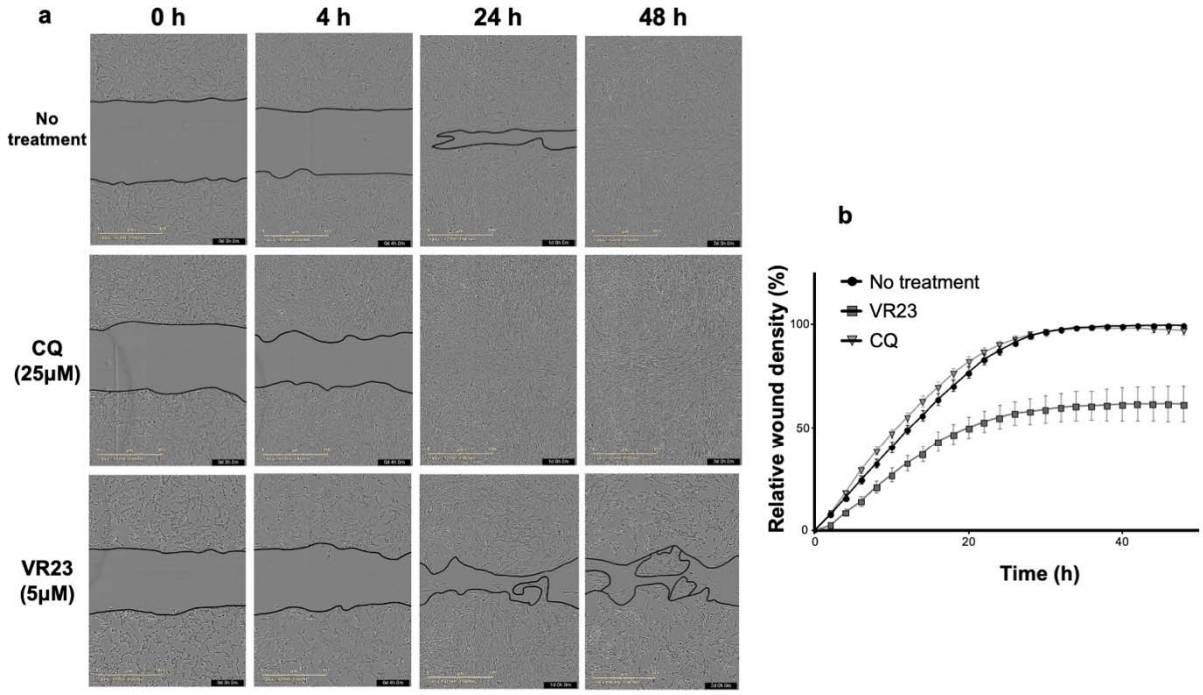

Figure 1.7. The results of the scratch wound assay of SW982 cells. (**a**) Images were taken using Incucyte S3 Live Cell Analysis System (10×) at every 2 h intervals (although shown only four time points). Scratch borders are outlined in black on the images. (**b**) Relative wound density calculated over 48-h time period.

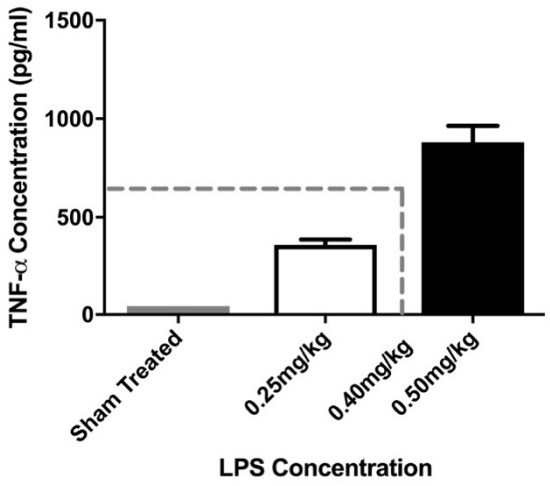

Figure 1.8. The optimal LPS concentration for the induction of TNF-α in the mouse lung was determined to be 0.4 mg/kg body weight.

Discussion

We previously showed that VR23 possesses strong anti-tumor activity *in vitro* and in animal models [157, 166]. VR23 is especially notable because it is not only non-toxic and preferentially kills cancer over non-cancer cells, but also shows strong synergy when combined with other therapeutic agents such as paclitaxel [166]. Here we report that VR23 also possesses strong anti-inflammatory property, which is consistent with the notion that the 7-chloro-4-aminoquinoline group renders anti-inflammatory property [177, 182]. The anti-inflammatory effect of VR23 is not specific to certain cell lines only, since it can reduce the response in a number of different model systems including monocyte cell lines, primary synoviocytes and the bronchoalveolar system in mice.

In our cell culture models, we found that VR23 substantially down-regulates the levels of IL-1β, TNF-α, IL-6, and IL-8, all of which are associated with inflammation [174, 183-186]. IL-6 is especially sensitive to VR23, suggesting that the IL-6 pathway may be a major target of VR23. Under conditions of acute stimulation, IL-6 can function as an anti-inflammatory cytokine [74]. However, overwhelmingly stimulated, IL-6 can be pro-inflammatory, leading to the development of chronic inflammation, autoimmune diseases, and cancer [187]. Data from our experiment with high doses of stimulants are likely translatable to the chronic inflammation caused by pro-inflammatory IL-6.

All of the four cytokines mentioned above critical inflammatory mediators that can induce damage even under acute inflammatory conditions and are intimately associated with the initiation of autoimmune conditions when unregulated for a prolonged period. The ability of VR23 to down-regulate all of these pro-inflammatory cytokines demonstrates its substantial potential for the treatment of both acute and chronic inflammatory conditions.

While VR23 effectively down-regulates four pro-inflammatory cytokines examined (i.e., TNF-α, IL-1β, IL-6, and IL-8), the BTZ and CFZ proteasome inhibitors do not show the same effects on the monocytic cells. It was previously suggested that proteasome inhibitors may down-regulate inflammation through the modulation of the nuclear factor-κB (NF-κB) pathway [159-161]. Bortezomib, in particular, was previously suggested to ameliorate inflammation if

administered prior to inflammatory induction. For example, Han and colleagues demonstrated that BTZ (25 nM or 50 nM) could reduce inflammation when administered prior to inflammation induction [188]. Similarly, Chen et al. demonstrated that BTZ at a dose of 25 nM down-regulated the inflammatory response in endotoxin-induced uveitis [161]. Contrarily to these reports, Hideshima et al. demonstrated that BTZ induces inflammatory response in multiple myeloma cells [162]. At least in part, the progression of multiple myeloma by BTZ could be due its ability to induce pro-inflammatory macrophages [163]. A study by Tilahun and colleagues also demonstrated the detrimental effects of BTZ in acute systemic inflammatory conditions such as sepsis [189]. Our data shown here clearly demonstrate that BTZ and CFZ do not notably down-regulate or up-regulate inflammatory cytokines at IC_{50} doses. The discrepancies discussed above may be stemmed from different doses and/or different cells used for each experiment. In any event, it is highly likely that the anti-inflammatory effect shown by VR23 is not a general property shared by proteasome inhibitors.

The anti-malarial HCQ is one of five non-biologic DMARDs. HCQ and its parental drug CQ were initially documented for their uses to treat autoimmune disorders such as RA and SLE [190], mainly due to their reported ability in down-regulating the levels of TNF-α, IL-6, and IL-1β in cells stimulated with endotoxin [191]. Today, HCQ is still used as a DMARD for RA due to its low toxicity profile, though it only has moderate clinical effects [192]. In the treatment of early RA, the use of non-biologic DMARD monotherapy is the recommended treatment by the American College of Rheumatology (ACR) guidelines [192]. If the patient has still high disease activity, combinations of methotrexate with HCQ or other DMARDs are recommended as initial therapy [192]. However, if the patient has poor prognostics with high disease activity, biologics such as anti-TNF agents are recommended [192]. Thus, DMARDs, including HCQ, remain essential treatment options for patients with RA even if more effective (and expensive) biological agents are available [192].

In our study, we found that CQ is effective only in down-regulating IL-6 in monocytes, and not effective on TNF-α, IL-6, and IL-8. This discrepancy may be due to the differences in CQ concentrations used. For example, Karres et al. [191] used 100-200 μM (which is 5-10 fold of IC_{50} values) while we used more clinically relevant doses (15 or 30 μM). Our data thus

indicate that VR23 would be more appropriate than CQ/HCQ for the treatment of inflammatory and autoimmune conditions. In particular, VR23 has the potential to be a highly effective agent for the treatment of RA, as it can not only effectively and preferentially down-regulate the level of IL-6 in HFLS-RA over HFLS-N, but also inhibit synovial cell migration (Figs 1.2 and 1.3). It should be noted that the dysregulation of IL-6 plays a large role in the development and progression of RA (and often other autoimmune conditions) [193]. IL-6 plays a critical role in the progression of RA; however, it is not the only factor involved in the manifestation and progression of RA. Rheumatoid arthritis is a complex disease, involving the dysregulation of many cytokines and factors. Nevertheless, the ability of VR23 to specifically down-regulate IL-6 in addition to other pro-inflammatory cytokines, making it an excellent candidate as a novel DMARD for RA treatments.

Our animal-based study clearly demonstrates that VR23 can be very effective for the treatment of ALI caused by LPS (and, potentially, by bacterial infection) (Fig. 1.4). When compared for efficacy alone, VR23 is similar to DEX, a glucocorticoid widely used at clinics. However, the prolonged use of corticosteroid hormone can lead to serious side effects on the musculoskeletal function, endocrine regulation, and cardiovascular and central nervous systems [194, 195]. Considering VR23 does not show any ill-effects to pre-clinical models [166], it would be better than DEX as a treatment agent against inflammation and autoimmune conditions.

Together with our previous reports [157, 166], data presented here suggest that VR23 is not only an effective and safe anti-tumor agent but also highly effective for the treatment of inflammation and autoimmune conditions, especially RA. Thus, VR23 can be a uniquely effective drug for cancer treatment as well as for the prevention of cancer development and progression caused by prolonged inflammation.

3.0 Chapter 2: The anti-inflammatory effect of VR23 is through the downregulation of the IL-6 mediated STAT3 signaling pathway

Abbreviations used in this article: BAF, bafilomycin; BTZ, bortezomib; CFZ, carfilzomib; CREB, cAMP-response element binding; CRP, c-reactive protein; DEX, dexamethasone; DMARD, disease-modifying anti-rheumatic drug; ELISA, enzyme linked immunosorbent assay; HCQ, hydroxychloroquine; HFLS, human fibroblast like synoviocytes; IL-6, interleukin-6; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; RA, rheumatoid arthritis; SOCS3, suppressor of cytokine signalling 3; STAT3, signal transducer and activator of transcription 3

Abstract

We previously demonstrated that VR23, a novel quinolone-sulfonyl compound with anticancer activity, downregulates IL-6 pro-inflammatory cytokine in response to LPS. To determine its functional mechanism, we examined if the anti-inflammatory effects of VR23 are through the STAT3, CREB, and/or SOCS3 pathways as they are all involved in the regulation of cellular inflammatory responses. STAT3 contains two pronounced phosphorylation sites, one on tyrosine 705 and the other on serine 727: the former residue is phosphorylated by JAK1 and the latter by PKC, MAPK and CDK5. We found that STAT3 phosphorylation on tyrosine 705 was substantially decreased in response to VR23, but not on serine 727. In addition, VR23 does not appear to involve in the regulation of JAK1, JAK2, CREB or SOCS3, indicating that its anti-inflammatory activity is not through IL-10 or IL-6/JAK1/JAK2. Thus, these data demonstrate that VR23 anti-inflammatory effect is specifically through the prevention of STAT3 phosphorylation on tyrosine 705. This conclusion was strengthened by the fact that: (a) the level of STAT3 upstream IL-6R was not affected, and (b) the expression of two STAT3 downstream genes, c-myc and MCP-1, were dramatically downregulated in response to VR23. Our data also showed that, unlike hydroxychloroquine, VR23 rapidly increased lysosomal pH. Taken together, our data are consistent with a model where VR23 targets STAT3 while promoting lysosomal pH to inhibit the amplification of the IL-6-STAT3 signaling pathway, resulting in strong anti-inflammatory effects.

Key words:

VR23; inflammation; rheumatoid arthritis; STAT3 pathway; IL-6; lysosomal acidity; cytokines; autoimmune; chloroquine; dexamethasone

Introduction

Interleukin 6 (IL-6) is a pleiotropic cytokine with important functions in the regulation of the immune system and inflammatory responses [196]. Thus, perhaps not surprisingly, its dysregulation may lead to the pathogenesis of various human diseases including rheumatoid arthritis (RA), juvenile chronic arthritis, Crohn's, psoriasis, and even cancers [187, 197-200]. Rheumatoid arthritis, a systemic and chronic inflammatory condition that leads to detrimental tissue damage [201], is characterized by an abnormal activation of T-lymphocytes, B-lymphocytes, mast cells, neutrophils, macrophages [202], and fibroblast-like synoviocytes [203]. It was shown previously that cells in the hyperplastic synovium in RA synovial joints express substantially elevated levels of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-8, IL-17, and IL-32 [204]. Among these cytokines, IL-6 has been widely implicated with the pathological progression of RA; where patients with active RA disease have elevated IL-6 levels both in serum [205] and synovial fluid [197]. The persistently high level of IL-6 can further drive synovial cell proliferation, leading to progressive joint destruction in RA patients [97, 206]. Since C-reactive protein (CRP) is produced by the liver in response to IL-6, it is widely used as a surrogate marker for IL-6 expression as well as for RA disease activity [100, 206-208].

The elevation of IL-6 in RA patients leads to the constitutive activation of STAT3, a critical player in the IL-6-JAK-STAT pathway important for cell proliferation and survival [68, 91]. STAT3 functions as a positive feedback loop driving high expression of inflammatory cytokines and receptor activator of nuclear factor kappa B ligand (RANKL). Furthermore, the phosphorylation of STAT3, which is correlated with the plasma concentration of IL-6, leads to an increase in the production of pro-inflammatory cytokines [68]. This in turn leads to even higher inflammation and greater joint destruction in RA patients. Therefore, the IL-6 mediated STAT3 pathway can be an effective target for the control of inflammatory and autoimmune conditions as well as many other human diseases including cancer [209].

Recently, tocilizumab, a monoclonal antibody targeting IL-6 receptor (IL-6R), has been developed for the treatment of autoimmune conditions including RA [196]. Data from clinical trials showed that tocilizumab is effective against several autoimmune conditions including RA [210]. In addition, several other monoclonal antibodies targeting IL-6 are in various stages of clinical trials, showing promises of this class of IL-6 targeting agents [211]. However, the monoclonal antibody-based treatment option has its own limit in a clinical setting as they are

generally expensive [212]; use invasive route of administration; and cause high rates of infections [213]. Thus, the development of novel inhibitors that are orally absorbed, fast in effectiveness, and low in toxicity are needed.

Currently, there are four common conventional disease-modifying anti-rheumatic drugs (DMARDs) available: methotrexate, sulfasalazine, hydroxychloroquine (HCQ), and leflunomide. Of these common DMARDs, none has been shown to specifically target IL-6 or IL-6R. In our previous study, we demonstrated that VR23, a novel 4-piperazinyl/amino quinolone derived sulfonyl compound with anticancer activity, has the ability to specifically reduce IL-6 in immune cell lines as well as in primary synoviocytes from RA patients [158, 214, 215]. We show in these papers that VR23 is not only safe and effective against tumors, but also possesses far greater anti-inflammatory effect than HCQ. In the current paper, we demonstrate that the anti-inflammatory effect of VR23 is through targeting the IL-6 mediated STAT3 pathway. The rest of the protocol was described previously [169, 170].

Materials and Methods

Reagents

VR23 was synthesized by Dalton Pharma Services (Toronto, Ontario, Canada). Bortezomib (BTZ), carfilzomib (CFZ), and cell-counting kit-8 (Enzo Life Sciences) were purchased through Cedarlane (Burlington, Ontario, Canada). The following reagents were purchased from Sigma Aldrich (Oakville, Ontario, Canada): dexamethasone (DEX), HCQ sulfate, LMT-28, LPS from *Escherichia coli* O111:B4, PCR primers, Phorbol 12-myristate 13-acetate (PMA), and soluble IL-6R. The following reagents were from Thermo Fisher Scientific (Waltham, MA): SYBR Green PCR Master Mix, ROX Reference Dye, LysoSensor Green DND-18, RPMI 1640, high-glucose DMEM, FBS, antibiotics and anti-mycotic solutions. The single analyte ELISA kits were from Qiagen (Montreal, Quebec, Canada). The following items were from R&D systems (Oakville, Ontario, Canada): IL-6, Human C-Reactive Protein Quantikine ELISA kit, phospho-STAT3 (Y705), phospho-STAT (s727) and total STAT3 antibodies. CREB, p-CREB, ERK and p-ERK antibodies and Monarch Total RNA Miniprep Kits were from Cell Signalling/New England Biolab (Whitby, Ontario, Canada). GAPDH antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). C-myc, SOCS3, and total (t)-IL-6R antibodies were from Abcam (Pleasanton, CA). IncuCyte pHrodo Green *E. Coli* Bioparticles were from Sartorius

Canada (Oakville, Ontario, Canada). RT2 First Strand Kit was from Qiagen (Montreal, Quebec, Canada). VR23 and control compounds were used at their respective IC_{50} concentrations as described previously [216], unless specified otherwise.

Cell lines and cell culture

THP-1 and SW-982 cells, purchased from the American Type Culture Collection, were cultured in RPMI 1640 (for the former) or high-glucose DMEM (latter) supplemented with 100 µg/mL streptomycin, 100 U/mL penicillin, and 10% (v/v) FBS. THP-1 and SW-982 cell lines were cultured at passages <10. J774A cell line was a kind gift of Dr. Hoang-Thanh Le (HSNRI, Ontario, Canada). Primary human fibroblast-like synoviocytes (HFLS) from three healthy donors (HFLS-NR) and two from RA patients (HFLS-RA) were purchased from Cell Applications (San Diego, CA) through Cedarlane. The cells were cultured in synoviocyte growth media and subcultured using reagents from the same supplier. The cells in each vial were from a single donor and provided at passage 1. All experiments with the HFLS-RA and HFLS-NR were conducted at passages 3-8, as previously described [167]. Primary human hepatocytes from three donors were from Sigma Aldrich (St. Louis, MO). Cells were thawed in Hepatocyte Thawing medium and used in Hepatocyte plating media for experiments. Both of the media were purchased from Sigma Aldrich. All experiments with the primary hepatocytes were conducted immediately after thawing.

ELISA

Human hepatocytes were plated at 0.7 x 10^6 cells/mL in a 0.5 mL volume for cytokine analysis. Quantikine ELISA was used for the measurement of CRP according to the supplier's recommendation (R & D system).

RT-qPCR

Total RNA was isolated from THP-1 cells (1.5 x 10^6 cells) with a Monarch Total RNA Miniprep Kit (New England Biolab). The isolated RNA was quantified on a Nanodrop spectrophotometer. RNA (3 µg) was reverse transcribed into cDNA with a RT2 First Strand kit (Qiagen). The resultant cDNA was used for quantitative (q)PCR with SYBR green master mix, Rox reference dye, and reverse and forward primers to measure the relative amounts of mRNA (i.e., gene

expression) in an AriaMx Real-Time PCR system. The forward (FH1) and reverse primer sequences (RH1) for c-myc and MCP-1 (Sigma Aldrich) are as follows: (FH1 MCP1) 5'-AGACTAACCCAGAAACATCC-3'; (RH1 MCP1) 5'-ATTGATTGCATCTGGCTG-3'; (FH1 c-MYC) 5'–TGAGGAGGAACAAGAAGATG-3'; and (RH1 c-MYC) 5'–ATCCAGACTGTGACCTTTTG-3'.

Western Blot Analysis

THP-1 cells were seeded in six-well plates at 0.5 x 10^6 cells /mL in 3 mL volume; and the HFLS cells were seeded in 10 cm plates at 0.05 x 10^6 cells /mL in 10 mL volume. The hepatocytes were seeded in six-well plates at 0.75 x 10^6 cells /mL in 2 mL. To examine phosphorylation status, THP-1 cells were incubated overnight at 37°C, treated with VR23 or control compounds for 1 h, and then stimulated with IL-6 (10 ng/mL) for 15 minutes. In the case of HFLS, cells were incubated overnight at 37°C, followed by treatment with VR23 or HCQ for 1 h, and then stimulated with IL-6 (100 ng/mL) and sIL-6R (100 ng/mL) for 30 minutes. The hepatocytes cells were incubated overnight at 37°C, treated with VR23 or HCQ for 1 h, and stimulated with IL-6 (50 ng/mL) for 15 minutes. To measure the total amounts of proteins, THP-1 cells were treated with VR23 and IL-6 (10 ng/mL) for 6 or 24 h. The cells were then treated with RIPA buffer for 30 minutes on ice, followed by triturating them using a syringe with a 25-gauge needle. The resultant cell lysates were resolved on a 10% (w/v) SDS-PAGE gel, followed by transfer to a nitrocellulose membrane. The membrane was blocked for 1 h in 5% (w/v) non-fat dry milk and then incubated overnight with appropriate primary antibodies against p-STAT3 (Y705) (1:400), p-STAT-3 (S727) (1:1000), p-ERK (1:1000), ERK (1:1000), c-myc (1:1000), IL-6R (1:1000), p-CREB (1:1000), CREB (1:1000), SOCS3 (1:1000), or GAPDH (1:10 000) in Tris-buffered solution (TBST) containing 5% (W/v) non-fat dry milk at 4°C. The membrane was washed three times (10 minutes each time) and incubated for 1 h with HRP-conjugated secondary antibodies diluted to 1:10 000. After 3 washes with TBST, the membrane was incubated with ECL select reagents, and chemiluminescent signals were visualized using a gel dock system and associate imaging program. The resultant bands were quantified by a densitometer.

Bioparticle Analysis

J774A cells were seeded at a density of 10,000 cells per well (50 µL/well) on a 96-well flat-bottom plate. The cells were incubated overnight to allow them to adhere to the plate. Serial dilutions of VR23 or HCQ (25 µL/well) were added at 4× final assay concentrations for 1 h. The Incucyte pHrodo Green Bioparticles from *E. coli* (25 µL/well) were added to each well at 10 µg/well. Live cell fluorescent imaging was captured every 10 minutes using an Incucyte Live-cell Imaging S3 system. Phagocytosis is quantified using the Incucyte analysis software (https://www.essenbioscience.com/en/applications/phagocytosis-bioparticles) by determining the green object area (acidic phagosomes) in the field of view over time. The area under curve from 0-12 h was then calculated for each dose of the compound in order to create a dose-response curve. This allows the pharmacology of VR23 and HCQ to be compared in terms of their ability to inhibit phagocytosis.

Immunofluorescence staining

SW-982 cells were seeded at a density of 62,500 cells per well on a 6-well plate. The cells were incubated overnight to allow adhere to the plate, followed by treating them with VR23, HCQ, or bafilomycin (BAF) for 6 h. Culture medium was replaced with fresh media containing lysosensor (Thermo Fisher) at 1 µM for 30 minutes. The cells were then imaged on the microscope.

Statistical analyses

Each experiment was repeated in three biological replicates unless otherwise indicated. The mean values of these results were used for statistical analysis and expressed as mean ± standard error. Comparison between experimental groups was made by *p* value determination using one-way ANOVA. The *p* value of <0.05 is considered to be statistically significant. Dunnett's post-hoc test was performed when necessary, to determine the significance between the treatment groups and controls. Analyses were performed using GraphPad Prism software, version 7.0e (San Diego, CA).

Results

VR23 prevents the phosphorylation of STAT3 in an LPS-induced cell model

Our previous study demonstrated that VR23 downregulates pro-inflammatory cytokines in response to LPS stimulation [214]. To further our previous work, we set out to examine the anti-inflammatory mechanism of VR23. We first examined if the anti-inflammatory effects of VR23 is through the STAT3, CREB, and/or SOCS3 pathways as they are all involved in the regulation of cellular inflammatory responses. As shown in Fig. 2.1a, VR23 decreased the phosphorylation of STAT3 on tyrosine 705 (Y705) by ~50% compared to the LPS-only sample by 6 h post-treatment. In contrast, carfilzomib (CFZ, a proteasome inhibitor like VR23) and dexamethasone (DEX, a well-known anti-inflammatory agent) did not show substantial reduction of STAT3 phosphorylation (Fig. 2.1a). These results suggest that VR23 targets the STAT3 activity while DEX and proteasome inhibitors in general do not.

To gain further insight into its specificity of targeting the STAT3 pathway, we examined if VR23 also affects the CREB and SOCS3 pathways. As shown in Fig. 2.1b, VR23, BTZ and CFZ did not significantly affect the phosphorylation of CREB, indicating that proteasome inhibitors do not upregulate CREB activity. The only exception was DEX, which upregulated CREB phosphorylation by approximately 40% (Fig. 2.1b). The phosphorylation of CREB protein leads to the induction of IL-10, which is known to inhibit the production of pro-inflammatory cytokines such as IL-6 in macrophage models [217-220]. We found that the SOCS3 protein levels are not substantially different between LPS-only and LPS+VR23 (Fig. 2.1c). SOCS3 is an inhibitor of IL-6 family signalling through directly inhibiting JAK1 and JAK2 [221]. Together data shown in Fig. 2.1 indicate that, unlike DEX, VR23 downregulates inflammation by directly targeting the STAT3 pathway, but not indirectly through targeting STAT1, STAT2, CREB or SOCS3.

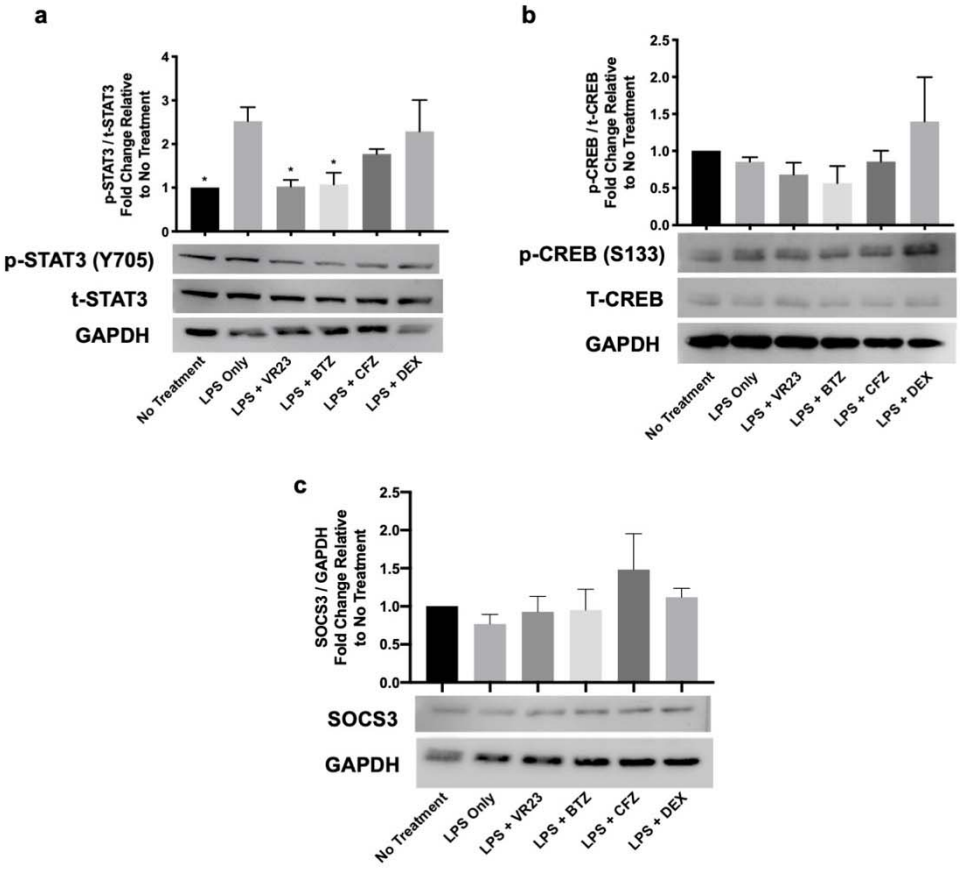

Figure 2.1 VR23, a known proteasome inhibitor, downregulates STAT3 activity in THP-1 cells. THP-1 cells were treated with VR23 or with various other compounds of related functionality at their respective IC_{50} concentrations for 6 h under the stimulation with LPS, followed by collection and analysis by Western blotting. (**a**) p-STAT3 (Y705), (**b**) p-CREB (S133) and (**c**) SOCS3 were detected by Western blotting with appropriate antibodies as described in Methods. For comparison, two other proteasome inhibitors (Bortezomib [BTZ, 6 nM] and carfilzomib [CFZ, 1 nM]), and one anti-inflammatory agent (dexamethasone [DEX, 2 mM]) were also included. Relative amounts of phosphorylated proteins were compared with those of respective total proteins and GAPDH (loading control). Fold change is displayed in relation to the no-treatment group. The comparison between samples was made using one-way ANOVA. The *p* values were determined by a Dunnett test, and the values presented are mean SEM (n = 3). *p<0.05, **p<0.01, and ***p<0.001, which denote significant differences from the LPS-only group. Note that *p* value is >0.05 when there is no asterisk. The values presented are mean ± SEM (n=3).

VR23 may downregulate STAT3 activity through IL-6 but not IL-6R

Having established that VR23 prevents STAT3 phosphorylation induced by LPS, we next determined whether the reduction of STAT3 phosphorylation by VR23 was due to a decrease in kinase activities, or indirectly through other signaling pathways. It was known that STAT3 can be activated through phosphorylation on the tyrosine 705 residue and on serine 727: the former mainly by JAK1 [222] and the latter by PKC, MAPK and CDK5 [223]. THP-1 cells were pre-treated for 1 h prior to IL-6 stimulation for 15 minutes as described previously [224]. We found that VR23 at 2.5 μM effectively decreased the phosphorylation of STAT3 on tyrosine 705 in a similar extent to LMT-28 in THP-1 cells (Fig. 2.2a). LMT-28 is an inhibitor of the IL-6-JAK-STAT pathway, through binding to the IL-6R, leading to the broad down-regulation of this pathway. Unlike VR23, none of the reference compounds, BTZ and CFZ proteasome inhibitors, DEX, or chloroquine (CQ) showed significant effects on the STAT3 phosphorylation on tyrosine 705 (Fig. 2.2a).

VR23 at 2.5 μM slightly decreased the phosphorylation of STAT3 on serine 727. However, this slight downregulation is considered not significant since LMT-28 also decreased the phosphorylation to a similar extent (Fig. 2.2b). None of the reference compounds showed any significant effect on the STAT3 phosphorylation on serine 727 (Fig. 2.2b). We then investigated whether VR23 inhibits PMA-induced ERK phosphorylation. We found that VR23 had little effects on ERK phosphorylation, even at 5 μM (Fig. 2.2c), indicating that VR23 is not directly involved in the regulation of the Raf-MEK-ERK pathway.

Since our data indicated that STAT3 is the target of VR23, we examined the effects of VR23 on the expression of c-myc as its transcription is induced by activated STAT3. VR23 at 2.5 μM downregulated c-myc expression by almost 80%, of which decrease is similar to that by LMT-28 (Fig. 2.2d). To determine more accurate functional point, we examined if VR23 has any effects on IL-6R, an upstream component of the IL-6-JAK-STAT3 signaling pathway (Fig. 2.2d, e). We found that at both 6- and 24-h post-treatment, neither VR23 nor LMT-28 downregulated the level of IL-6R. Together these results demonstrate that VR23 targets STAT3, but probably not the upstream components of the IL-6-JAK-STAT3 pathway, although we cannot completely rule it out (see Discussion).

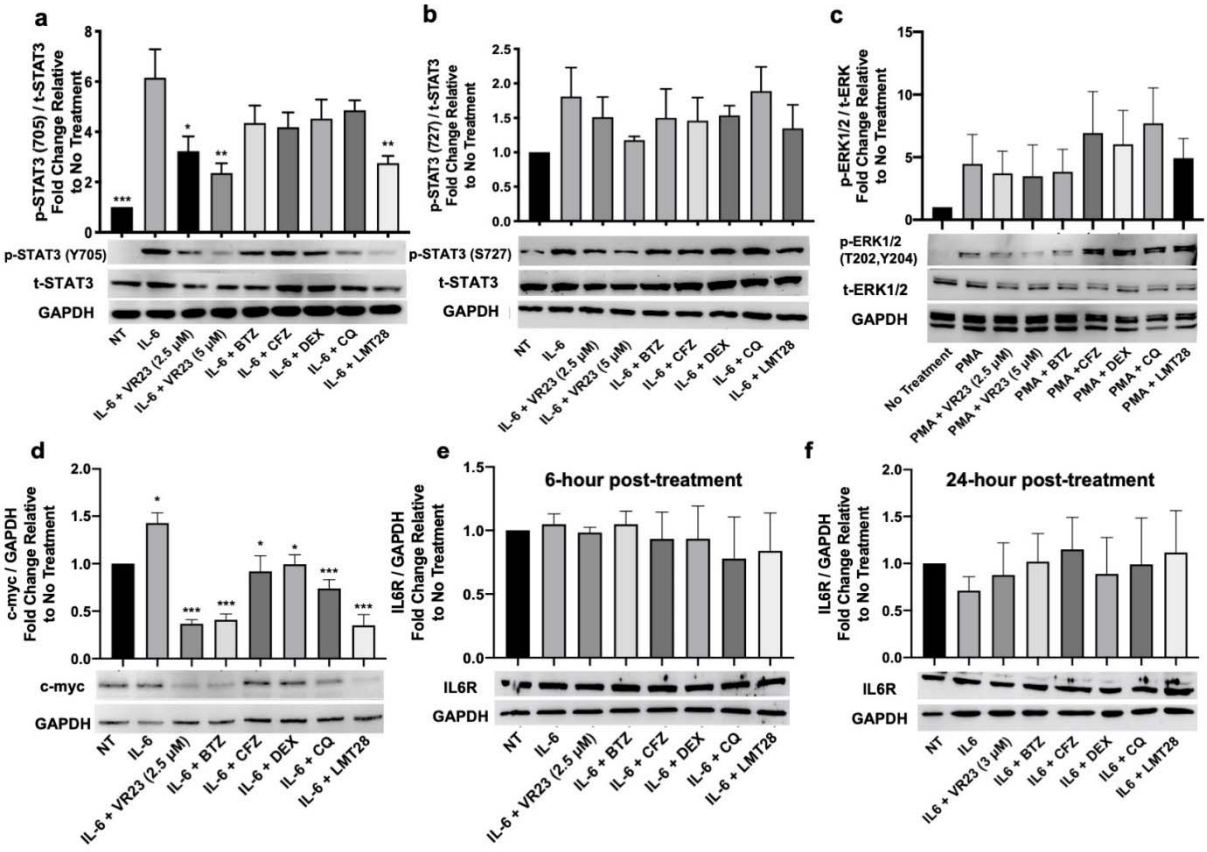

Figure 2.2. Blockade of IL-6 signaling by VR23 is through the inhibition of STAT3 in THP-1 cells. THP-1 cells were treated with VR23 or various other compounds of related functionality at their respective IC_{50} concentrations for 1 h prior to stimulation with IL-6 (10 ng/mL) or PMA (100 nM) for 15 minutes, followed by Western blot analysis. Lysates were analyzed for (**a**) p-STAT3 (Y705), (**b**) p-STAT (S727), and (**c**) p-ERK1/2 (T202, Y204). THP-1 cells were treated similarly but for 6 h or 24 h under the stimulation with IL-6 (10 ng/mL). Lysates were analyzed for c-myc (**d**), or IL-6R (**e, f)** at 6-h (**e**) or 24-h (**f**) post-treatment. For comparison, BTZ (6 nM), CFZ (1 nM), DEX (2 mM), chloroquine (CQ; 25 μM), and LMT-28 (40 μM) were also included. Fold change is displayed in relation to the no-treatment control. The comparison between groups was made using one-way ANOVA. The *p* values were determined by a Dunnett test, and the values presented are mean SEM (n = 3). *p<0.05, **p<0.01, and ***p<0.001, which denote significant differences from the IL6-only or PMA-only group. Note that *p* value is >0.05 when there is no asterisk. The values presented are mean ± SEM (n=3).

VR23 reduces gene expression of IL-6/STAT3 signaling components

To understand the full effects of VR23 on the IL-6-JAK-STAT signaling, the mRNA levels of important downstream genes were analyzed by RT-qPCR. In response to VR23, the mRNA levels of c-myc proto-oncogene and monocyte chemoattractant protein-1 (MCP-1) were decreased by 4-fold and 12-fold, respectively (Fig. 2.3a, b). Interestingly, DEX downregulated MCP-1 by approximately 30-fold, of which implication is currently unclear.

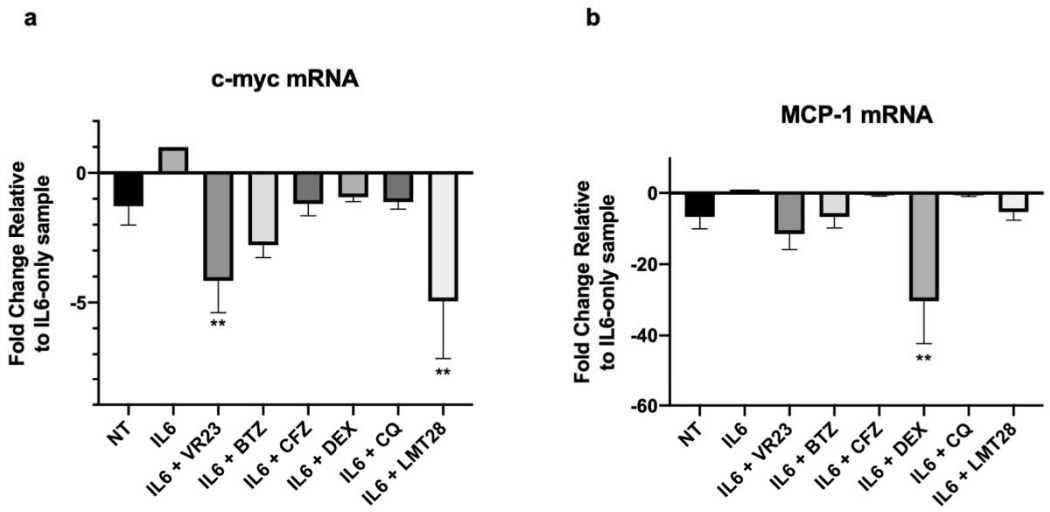

Figure 2.3. VR23 downregulates the expression of c-myc (**a**) and MCP-1 (**b**) genes that function downstream of IL-6. The relative levels of mRNA of indicated genes were quantified through RT-qPCR. Quantification of relative gene expression levels in fold change was calculated using ΔΔCt, and then normalized the fold change to the IL-6-only samples. The inverse of the fold change here is shown to illustrate the downregulation of gene expression. For comparison, BTZ (6 nM), CFZ (1 nM), DEX (2 mM), CQ (25μM), and LMT-28 (40 μM) were also included. The comparison between groups was made using one-way ANOVA. The *p* values were determined by a Dunnett test, and the values presented are mean SEM (n = 3). *p<0.05, **p<0.01, and ***p<0.001, which denote significant differences from the IL6-only group. Note that *p* value is >0.05 when there is no asterisk. The values presented are mean ± SEM (n=3).

VR23 prevents STAT3 phosphorylation in human primary fibroblast-like synoviocytes (HFLS) and hepatocytes.

Data in Fig. 2.1 showed that VR23 suppresses STAT3 activation in the THP-1 cell line. We next examined whether VR23 also similarly suppresses STAT3 activity in primary human cells with chronic inflammation. For this study, soluble IL-6R (sIL-6R) and IL-6 were used as stimulants to mimic a microenvironment similar to an inflamed RA joint. We found that VR23 was effective in reducing the phosphorylation of STAT3 (Y705) to the no treatment background level in HFLS cells from RA patients (Fig. 2.4a). VR23 also prevents STAT3 phosphorylation induced by IL-6/sIL-6R in HFLS-NR (normal) cells to a similar extent (Fig. 2.4b). In contrast, HCQ, a common DMARD used for treating RA was not effective in downregulating p-STAT3 in HFLS cells from either healthy donors or RA patients (Fig. 2.4).

C-reactive protein is a protein of hepatic origin found in blood plasma, whose circulating concentrations rise in response to inflammation following IL-6 secretion by macrophages and T cells [225-227]. CRP is a vital component of the acute phase response and high levels have been found in patients with high disease activity of RA patients [228, 229]. Therefore, we examined the levels of CRP in primary hepatocytes. In the experiment, we first examined the phosphorylation levels of STAT3 to confirm that STAT3 activation is also controlled by IL-6 in hepatocytes. As shown in Fig. 2.5a, VR23 was effective in reducing STAT3 phosphorylation on tyrosine 705 by approximately 45%, compared to the IL-6 only control. As anticipated, the CRP level in the hepatocyte supernatant was also mildly decreased in response to VR23 (Fig. 2.5b). Interestingly, HCQ was as effective as VR23 in reducing the level of STAT3 phosphorylation on tyrosine 705 in hepatocytes (Fig. 2.5a) but was substantially less effective in reducing the level of hepatocyte CRP (Fig. 2.5b).

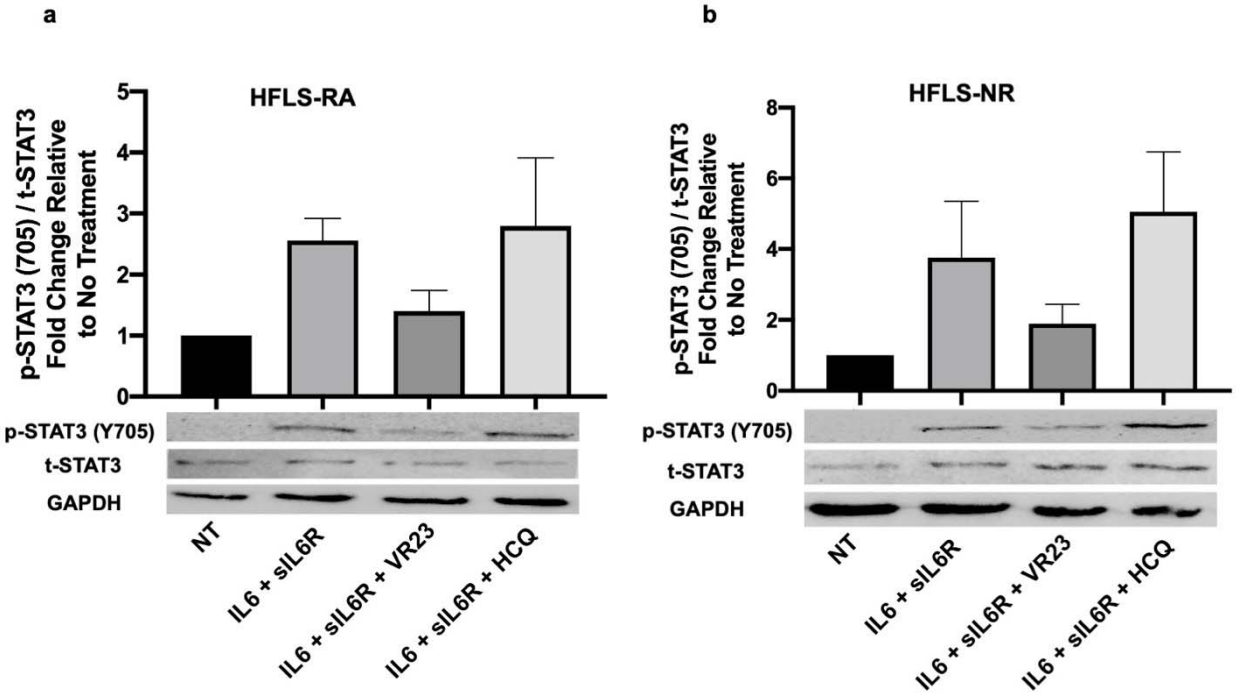

Figure 2.4. VR23 prevents the STAT3 phosphorylation induced by IL-6/sIL-6R in HFLS cells from both healthy donors and RA patients. (**a**) Primary RA (HFLS-RA) and (**b**) normal (HFLS-NR) synoviocytes were treated with VR23 (5 μM) or HCQ (30 μM) for 1 h prior to stimulation with sIL-6R (100 ng/mL) + IL-6 (100 ng/mL) for 30 minutes. Lysates were then analyzed for p-STAT3 (Y705) levels. The levels of phosphorylated STAT3 were compared with those of total proteins and the GAPDH loading control. Fold change is displayed in relation to the no-treatment control. The comparison between samples was made using one-way ANOVA. Note that *p* value is >0.05 when there is no asterisk. The values presented are mean ± SEM (n=3).

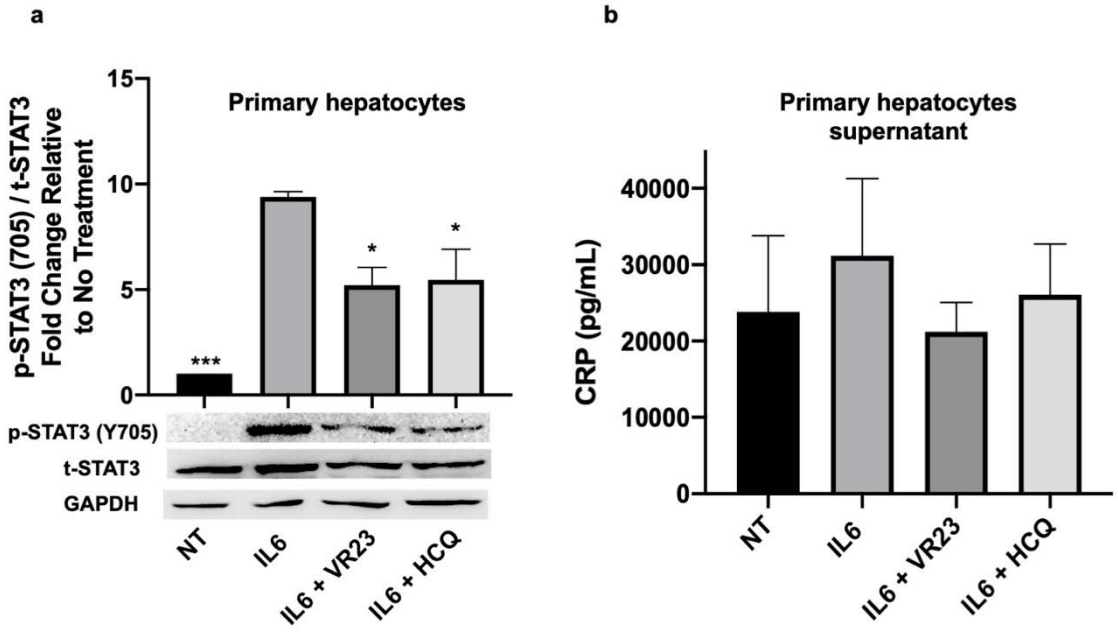

Figure 2.5. VR23 downregulates the STAT3 pathway through decreasing phosphorylation on tyrosine 705 and the level of C-reactive protein (CRP) in the IL-6-stimulated primary hepatocytes. (**a**) Primary hepatocytes were treated with VR23 (5 μM) or HCQ (30 μM) for 1 h, stimulated with IL-6 (50 ng/mL) for 15 minutes, and then collected to analyze the level of p-STAT3 (Y705) and total STAT3 (t-STAT3) proteins. GAPDH was used as a loading control. Fold change is displayed in relation to the no-treatment control. The comparison between samples was made using one-way ANOVA. The *p* values were determined by a Dunnett test, and the values presented are mean SEM (n = 3) *$p< 0.05$, **$p<0.01$, and ***$p<0.001$ denote significant differences from the IL-6 group. (**b**) Shown are the levels of CRP secreted in the supernatant of primary hepatocytes stimulated with IL-6 (50 ng/mL) and then treated with VR23 or HCQ at their respective IC_{50} values for 6 h. CRP levels were measured using an ELISA. The comparison between samples was made using one-way ANOVA. The *p* values were determined by a Dunnett test, and the values presented are mean SEM (n = 3). Data shown in panel b are $p>0.05$.

VR23 strongly disrupts lysosomal acidity.

STAT3 is known to coordinately regulate endocytosis, intracellular trafficking, and lysosome biogenesis, in addition to its regulation on inflammatory response [230]. We previously found that both VR23 and HCQ localize in the lysosome [141, 231]. This raises the possibility that both VR23 and HCQ may downregulate STAT3 activation through the lysosome signal. The anti-inflammatory capability of chloroquine, specifically its ability to reduce IL-6, has been attributed to its ability to alter the endosomal and lysosome pathways [232, 233]. Since maintaining acidity (pH 4.8-5.0) is important for normal lysosomal functions, we examined whether lysosomal pH is altered in response to VR23 or HCQ. Toward this study, we first measured the IC_{50} values (inhibiting cell growth by 50%) of both VR23 and HCQ in J774A macrophage cells, which were 23-24 μM range for both (Fig. 2.6a). Next, we studied the ability of VR23 and HCQ in reducing the acidity of phagosome/lysosomes using bioparticles which increase fluorescence in an acidic environment. The data are then shown as an area under curve for data points collected from 0-12 h with varying concentrations of the compounds (Fig. 2.6b), from which phagocytosis inhibition IC_{50} values are determined (Fig. 2.6c). The phagocytosis IC_{50} values demonstrated that VR23 is able to increase lysosomal pH by a dose 4.6 times lower than HCQ (8.85 μM *vs* 41.45 μM) even though their IC_{50} values for cell growth were comparable. This demonstrates the superior ability of VR23 to effectively alter lysosomal pH.

To gain further insights into VR23 effects on autoimmune-related inflammation, we examined the alterations of lysosome acidity in the SW-982 human synovial cells [173]. Cells were treated for 6 h with either VR23, HCQ, or BAF, a V-ATPase inhibitor known to increase lysosomal pH [234]. The resultant data showed that both VR23 and BAF effectively reduced the fluorescent punctae of the lysosomes, indicating that they effectively increased pH. In contrast, HCQ was not effective on increasing lysosomal pH (Fig. 2.6d, e). These data indicate that the anti-inflammatory effects by VR23 and HCQ are not the same and could demonstrate why VR23 is much more effective than the latter in downregulating inflammation.

a

IC_{50} values (μM) on J774A	
VR23	23.41 ± 7.94
HCQ	24.42 ± 3.03

c

Phagocytosis inhibition IC_{50} value (μM), from panel b	
VR23	8.85
HCQ	41.45

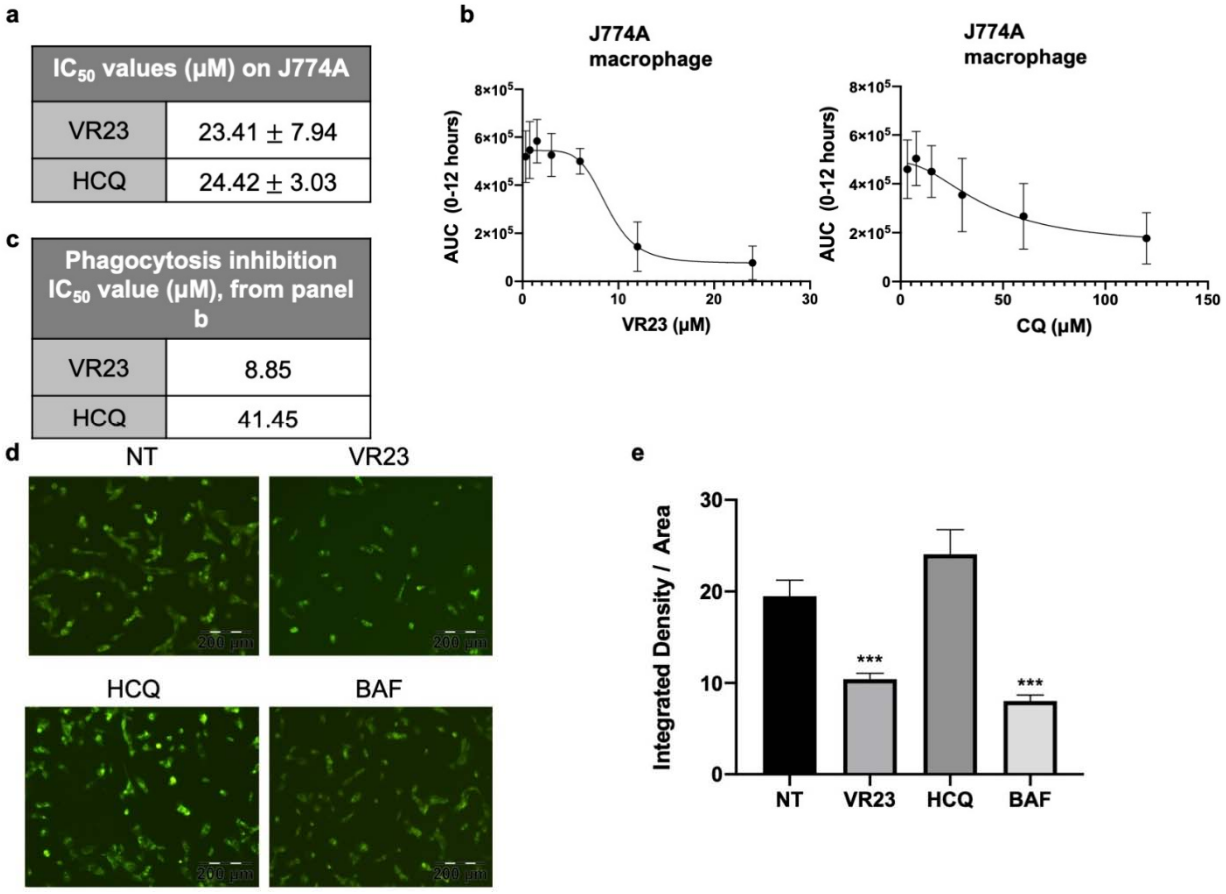

Figure 2.6. VR23 may disrupt lysosomal acidification. (**a**) IC_{50} values of VR23 and HCQ on J774A macrophages are shown. (**b**) Data collected from Incucyte software is displayed as area under curve for 0-12 h to determine the ability of the compounds to inhibit phagocytosis using a dose curve. Area under curve is a cumulative measurement of drug effect in pharmacokinetics. (**c**) Phagocytosis inhibition IC_{50} values determined from data in panel **b**. (**d**) SW982 cells were treated with VR23, HCQ, or BAF at their respective IC_{50} values for 6 h, followed by incubation with lysosensor for 30 minutes prior to imaging. (**e**) Integrated density of cells in the microscopic images shown in panel **d**, normalized to the area of the cells and to background. The comparison between samples was made using one-way ANOVA. The *p* values were determined by a Dunnett test, and the values presented are mean SEM (n = 3). **p*< 0.05, ***p*< 0.01, and ****p*<0.001 denote significant differences from the no-treatment control.

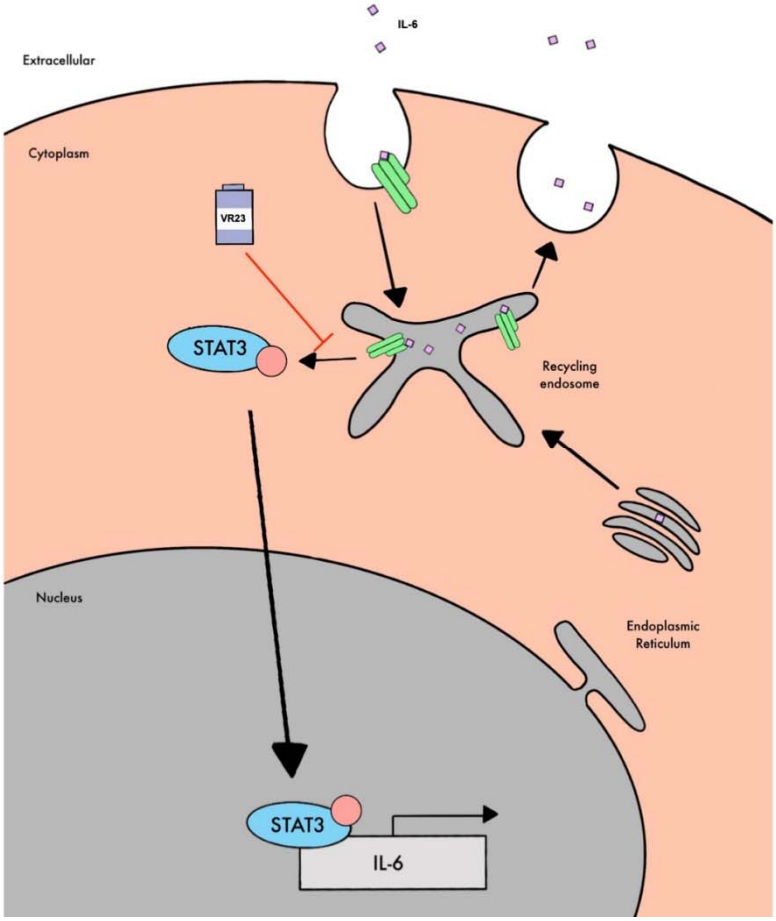

Figure 2.7. Illustration of potential anti-inflammatory mechanism of VR23. VR23 inhibits the production of IL-6 from cells, as shown previously [214]. Data in this paper demonstrate that VR23 can reduce p-STAT3 through IL-6 signalling, leading to the downregulation of STAT3 dependent genes such as MCP-1, c-myc, and CRP. Thus, our data are consistent with the model that VR23 is eliciting anti-inflammatory effects through altering the pH of the endosome, thereby preventing further amplification of the IL-6-STAT3 signal.

Discussion

We previously showed that VR23 downregulates IL-6, which was thought relevant to its strong anti-inflammatory property [214]. Here we report that the anti-inflammatory mechanism of VR23 is through the downregulation of the IL-6 mediated STAT3 pathway. First, we showed that, unlike other proteasome inhibitors such as BTZ and CFZ or the corticosteroid DEX, VR23 effectively downregulated the STAT3 activity by preventing its phosphorylation on tyrosine 705 (Fig. 2.1a). However, unlike DEX, VR23 did not upregulate the phosphorylation of CREB that induces IL-10 (Fig. 2.1b). Since IL-10 can inhibit the production of pro-inflammatory cytokines such as IL-6, this data indicates that the anti-inflammatory effect of VR23 may directly target STAT3. In contrast, DEX may downregulate inflammation through the IL-10 pathway. Further, VR23 did not affect SOCS3, an IL-6 inhibitor that works by directly binding to JAK1 and JAK2, but not JAK3 [221]. Thus, our data are consistent with the notion that the VR23-mediated downregulation of the IL-6-STAT3 activity may not be through the JAK pathway. However, the effects of VR23 on the JAK1-STAT3 pathway may need further investigation to confirm this tentative conclusion. In contrast to VR23, CFZ, another proteasome inhibitor, upregulated SOCS3 (Fig. 2.1c). Together, these data demonstrate that VR23 may downregulate inflammation by directly targeting the IL-6-STAT3 pathway, but not through the STAT1, STAT2, CREB, or SOCS3 pathway. Our data also suggest that the VR23 anti-inflammatory effect is independent of its proteasome inhibitor function.

In response to IL-6 stimulation, 2.5 μM VR23 effectively prevented STAT3 phosphorylation on tyrosine 705 in a similar extent to LMT-28, a specific inhibitor of the IL-6 mediated JAK-STAT pathway (Fig. 2.2a). Under the same conditions, however, VR23 did not effectively downregulate STAT3 phosphorylation on serine 727 (Fig. 2.2b), or ERK1/2 (Fig. 2.2c). These data demonstrate that the VR23-mediated downregulation of STAT3 activity is indeed through the IL-6-STAT3 pathway, but not through the PKC, MAPK or CDK5 pathway. This conclusion is further strengthened by the fact that the IL-6-STAT3 induced expression of c-myc was effectively downregulated by a low dose VR23 (Figs. 2.2d, 2.3a). Unlike the expression of c-myc, the level of IL-6R was not downregulated at least up to 24 h of post-treatment (Fig. 2.2e, f). This data may indicate that VR23 specifically targets IL-6-STAT3, but not through IL-6R-STAT3 signal. However, with our data alone, we cannot completely rule out the possibility

that VR23 downregulates the IL-6-STAT3 pathway at the IL-6R endocytosis step or through the gp130 subunit. Since DEX and HCQ had little effects on the STAT3 phosphorylation on tyrosine 705 (Fig. 2.2), VR23 possesses a unique mechanism of action in the downregulation of inflammation among well-known anti-inflammatory agents.

The chemotactic cytokine MCP-1 has been implicated in the pathological progress of RA. MCP-1 levels are higher in synovial fluid and sera from RA patients compared to synovial fluid from osteoarthritis patients [105]. We previously showed that VR23 effectively downregulates the level of MCP-1 in HFLS cells [214]. Our data shown here demonstrate that the VR23-mediated downregulation of MCP-1 is at the level of transcription regulated by IL-6 (Fig. 2.3b), which is consistent with the notion that VR23 functional point is at IL-6, leading to the downregulation of STAT3 and its controlled genes. Our data thus suggest that VR23 targets IL-6-STAT3 mediated genes, leading to a downregulation of the IL-6-STAT3 mediated signal amplification.

Data from our bioparticle and microscopic assays clearly demonstrate that VR23 is effective at increasing the pH of the lysosomes. When compared for pH altering abilities, VR23 showed similar efficacy to BAF, an antibiotic well-known for its ability to raise the pH of acidic vacuoles. It may be important to note that STAT3 coordinately regulate endocytosis, intracellular trafficking, and lysosome biogenesis [230]. In this context, our data appear to indicate that VR23 disrupts pH in relatively short time, thereby preventing the IL-6 uptake in the cell, leading to the downregulation of the phosphorylation of STAT3. Overall, our data are consistent with a model where VR23 inhibits the IL-6 mediated STAT3 pathway by targeting the endosomal axis, by which the expression of genes controlled by the pathway is downregulated, and the further signal amplification of the IL-6-STAT3 pathway is inhibited, resulting in strong anti-inflammatory effects by VR23 (Fig. 2.7).

Contrary to many studies, HCQ did not substantially increase the pH of the acidic vacuoles in the bioparticle assay or the lysosensor microscopy. However, our data is consistent with a recent study [235]. The discrepancy may be, at least in part, due to the length of HCQ treatment duration used in different experiments since lysosomal pH increase by HCQ is not an

active mechanism but caused mainly by the accumulation of protonated HCQ molecules in the lysosome.

RA is a chronic disease that is characterized by joint inflammation and destruction [236] promoted by elevated IL-6 activities [237]. RA patients have shown to have increased STAT3 activities in synovial tissues [90, 238]. HCQ remains to be one of four non-biologic DMARDs used for the treatment of autoimmune-related diseases including RA. Despite the availability of other options developed more recently, HCQ remains a common treatment option due to a variety of reasons including the high cost of antibody-based treatments [192]. However, our data demonstrate that HCQ is not effective at downregulating STAT3 activity in synoviocytes (Figs. 2.1, 2.4), MCP-1 (Fig. 2.3 & [214]), phagocytosis (Fig.2.6), or other anti-inflammatory effects [214]. In contrast, VR23 specifically downregulates the IL-6 mediated STAT3 pathway with high anti-inflammatory effects [214]. Therefore, VR23 has an excellent potential as an anti-RA agent, and perhaps for the control of other autoimmune-related chronic inflammatory diseases.

4.0 Chapter 3: The identification of VR23 metabolites and their activity as anti-inflammatory agents

* Mass spectrometry data were generated by Dr. James Knockleby, a Research Associate at the Lee laboratory.

Abbreviations used in this article: BAF, bafilomycin; DMARD, disease-modifying anti-rheumatic drug; ELISA, enzyme linked immunosorbent assay; HCQ, hydroxychloroquine; HFLS, human fibroblast like synoviocytes; IL-6, interleukin-6; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; RA, rheumatoid arthritis; STAT3, signal transducer and activator of transcription 3

Abstract:

I previously demonstrated that the VR23-mediated downregulation of IL-6 pro-inflammatory cytokine is likely through the prevention of STAT3 phosphorylation on tyrosine 705. The data also indicated that the downregulation is independent of proteasome inhibition activity although VR23 is a proteasome inhibitor targeting β2 subunit of the 20S proteasome catalytic unit. Interestingly, I noted during our study of its anti-inflammatory potential that VR23 rather rapidly metabolizes in human cells, raising a possibility that the VR23-mediated anti-inflammatory effects could be by the metabolite molecules not by VR23 *per se*. I therefore systematically investigated VR23 metabolites. We found that 7-chloro-4-(piperazin-1-yl)quinoline (CPQ) and 4-((4-(7-chloroquinolin-4-yl)piperazin-1-yl)sulfonyl)-3-nitroaniline (DK23) are two major metabolites. Among these two, only DK23 was active and possessed the ability to downregulate pro-inflammatory cytokines in human synovial cells. I found that DK23 downregulated STAT3 phosphorylation on tyrosine 705 at a similar extent to its parental VR23. Also, similarly to VR23, DK23 rapidly increased lysosomal pH. Taken together, the data are consistent with the notion that VR23 is metabolized in the cells to produce DK23, which in turn functions as a strong anti-inflammatory agent. This data also indicates that VR23 is a prodrug in downregulating the inflammatory response.

Introduction

Disease modifying anti-rheumatic drugs (DMARDs) are often used for autoimmune conditions such as rheumatoid arthritis. One important aspect of using these drugs is to understand how they are metabolized in the body. An active metabolite may form when a drug is metabolized into a modified form which elicits its effects in the body [239]. The pharmacological effects of metabolites are often similar to that of the parent drug, although they can also be toxic in certain cases [240].

In the treatment of rheumatoid arthritis, there are four conventional DMARDs that are commonly used to control the disease: leflunomide, sulfasalazine, methotrexate, and hydroxychloroquine. The main active metabolite of leflunomide is teriflunomide, which accounts for 70% of leflunomide after conversion in the body [241]. Leflunomide and teriflunomide have the same pharmacological mechanism in their immunosuppressive effects [241, 242]. Sulfasalazine, another DMARD, is 90% metabolized upon reaching the colon where its converted into sulfapyridine and mesalazine [118, 243]. Both metabolites are active, however mesalazine remains in the colon while sulfapyridine is further broken down to N-acetylsulfapyridine [118, 243]. Hydroxychloroquine and other 4-aminochloroquines are metabolized in the liver by dealkylation leading to the production of two metabolites, desethyl chloroquine and bisdesethyl chloroquine [244]. Approximately 30-80% of hydroxychloroquine is typically metabolized, while the rest is excreted [245-247]. Both metabolites have similar pharmacological activity as their parent molecules [244]. Lastly, methotrexate is a commonly used DMARD for RA, however it does not have an active metabolite, rather it acts as an unchanged drug, with a small percentage of it converted to 7-hydroxymethotrexate [248].

Our novel anti-inflammatory agent, VR23, has previously demonstrated potent activity in reducing pro-inflammatory cytokines in both acute and chronic inflammatory models. Further, VR23 has demonstrated the ability to regulate the IL-6-STAT3 axis, by preventing the phosphorylation of STAT3 under inflammatory conditions. Specifically, we have demonstrated VR23's ability to reduce inflammation and p-STAT3 in human fibroblast like synoviocytes (HFLS) from rheumatoid arthritis patients, giving it substantial potential as a therapeutic for rheumatoid patients who have not experienced effective disease reduction with the conventional

DMARDs. To further characterize the drug and understand how it may elicit these effects in humans, I examined the existence of VR23 metabolites. I found that VR23 rapidly metabolized into two different compounds, one of which is as active as VR23, and the other not active at all.

Materials and Methods

Reagents

VR23 [141] (Dalton Pharma Services, Toronto, Ontario, Canada) was prepared in DMSO to a stock solution of 20 mM. HCQ sulfate (>98%) and CPQ were purchased from Sigma Aldrich (Oakville, ON) and solubilized with water and DMSO respectively. DK23 was synthesized by Toronto Research Chemicals (Toronto, ON, Canada) and its stock solution (20 mM) was prepared in DMSO. The following items were purchased from Thermo Fisher Scientific (Waltham, MA): IL-1β, RPMI 1640, high glucose DMEM, FBS, and antibiotic and antimycotic solution. The IL-1β single analyte ELISA kit was purchased from Qiagen (Montreal, Quebec, Canada). The following items were purchased from R&D systems (Oakville, Ontario, Canada): IL-6, MCP-1 Protein Quantikine ELISA kit, IL-6 Protein Quantikine ELISA kit, phospho-STAT3 (Y705), and total STAT3 antibodies. GAPDH antibody was purchased from Origene.

Cell lines and Cell culture

THP-1 and SW-982 cell lines were purchased from the American Type Culture Collections (ATCC) and cultured in RPMI 1640 (former) and high-glucose DMEM (latter) supplemented with 100 μg/mL streptomycin, 100 U/mL penicillin, and 10% (v/v) FBS. The THP-1 cells were used for experiments at passages <10. Primary human fibroblast-like synoviocytes from healthy donors (HFLS-N) and donors with RA (HFLS-RA) were purchased from Cell Applications (San Diego, CA) through Cedarlane (Burlington, Ontario, Canada). The cells were cultured according to suppliers' instruction, using synoviocyte growth medium from the supplier. The cells in each vial were isolated from a single donor and all experiments were conducted at passages 3-8, as described previously [167].

ELISA

THP-1 cells were plated at a density of 500,000 cells/ml in 1 ml volume and HFLS were plated at 50,000 cells/ml in 0.5 ml for cytokine analysis. Single Analyte ELISA kit (Qiagen) was used

for IL-1β as per supplier's recommendations. Quantikine ELISA kits for each of the individual cytokines, MCP-1 and IL-6, (R&D Systems) were also used following recommendations by the supplier.

Western Blotting

To examine phosphorylation status, HFLS cells were incubated overnight at 37°C, followed by treatment with VR23 or HCQ for 1 h, and then stimulated with IL-6 (100 ng/mL) and sIL-6R (100 ng/mL) for 30 minutes. The cells were treated with RIPA buffer for 30 minutes on ice, followed by lysing with trituration using a syringe with a 25-gauge needle. The resultant cell lysates were resolved on a 10% (w/v) SDS-PAGE gel, followed by transfer to a nitrocellulose membrane. The membrane was blocked for 1 h in 5% (w/v) non-fat dry milk and then incubated overnight with appropriate primary antibodies against p-STAT3 (Y705) (1:400), t-STAT-3 (1:1000), or GAPDH (1:10 000) in Tris-buffered solution (TBST) containing 5% (w/v) non-fat dry milk at 4°C. The membrane was washed three times (10 minutes each time) and incubated for 1 h with HRP-conjugated secondary antibodies diluted to 1:10 000. After 3 washes with TBST, the membrane was incubated with ECL select reagents, and chemiluminescent signals were visualized using a Fluorochem Q gel doc system and associate AlphaView (version number) imaging program. The resultant bands were quantified by a densitometer.

Liquid chromatography mass spectrometry

Cells treated with VR23 were lysed in ddH_2O by sonication, and then cleared with an equal amount of acetonitrile. The lysates were centrifuged at 13,000g for 10 minutes and supernatant collected into HPLC insert vials and stored at -20°C until analysed. Analytes were measured on a Waters Xevo G2-XS Quadrupole Time-of-Flight mass spectrometer coupled to a Water Acquity Class I UHPLC (Laurentian University; Perdue Central Analytical Facility). A 10 cm Waters ACQUITY UPLC BEH (ethylene bridged hybrid) C18 130Å, 1.7 µm, 2.1 mm X 100 mm Column, coupled to a Waters ACQUITY UPLC BEH C18 VanGuard 130Å, 1.7 µm, 2.1 mm X 5 mm Pre-column was used to separate analytes, using a ballistic gradient from 70:30 H_2O:ACN to 20:80 H_2O:ACN over the course of 10 minutes. Leucine Enkephalin was used as a lock mass and sodium formate was used to calibrate the mass spectrometer. Standards were dissolved in 50:50 ddH_2O:ACN from a DMSO stock. All reagents used were HPLC grade.

Microscopy

SW-982 cells were seeded at a density of 62,500 cells per well on a 6-well plate. The cells were incubated overnight to allow adherence to the plate, followed by treatment with the compounds: DK23, VR23, HCQ, or bafilomycin (BAF) for 6 h. Culture medium was replaced with fresh media containing Lysosensor (Thermo Fisher) at 1 μM for 30 minutes. The cells were then imaged on the microscope.

Statistical analyses

Experiments that were repeated in three biological replicates were analyzed for their statistical differences. The mean values of these results were used for statistical analysis and expressed as mean ± standard error. Comparison between experimental groups was made by *p* value determination using one-way ANOVA. The *p* value of <0.05 is considered to be statistically significant. Dunnett's post-hoc test was performed when necessary, to determine the significance between the treatment groups and controls. Analyses were performed using GraphPad Prism software, version 7.0e (San Diego, CA).

Results

Determination of VR23's metabolites

Our previous studies demonstrated that VR23 is anti-inflammatory for both acute and chronic inflammatory conditions [214]. As a potential new DMARD, it is important to know whether VR23 is metabolized *in vivo* as most of the current DMARDs for RA are metabolized into active compounds in human cells. We found that VR23 is also metabolized rather quickly in human cells. We first examined VR23 treated SW982 synovial cells by LCMS, to determine if metabolites of VR23 were present or if VR23 remained as the only active molecule in the cell. We found that VR23 is metabolized into two main compounds (Fig. 3.1a). Drug metabolism usually occurs in hepatic cells through the CYP450 system [249], so it was surprising to see the metabolism of VR23 into two different compounds in the synovial cells. To identify the metabolites, we performed electrospray ionization mass spectrometry coupled with liquid chromatography. Based on the predicted masses of the ions and collision induced disassociation (CID) fragmentation, we predicted the structures of the two compounds. To confirm their identities, we obtained those compounds predicted by the analysis of metabolites: CPQ, an

already known compound, was purchased from Sigma Aldrich, and the novel DK23 compound was synthesized through the Toronto Research Chemicals (Fig. 3.1b).

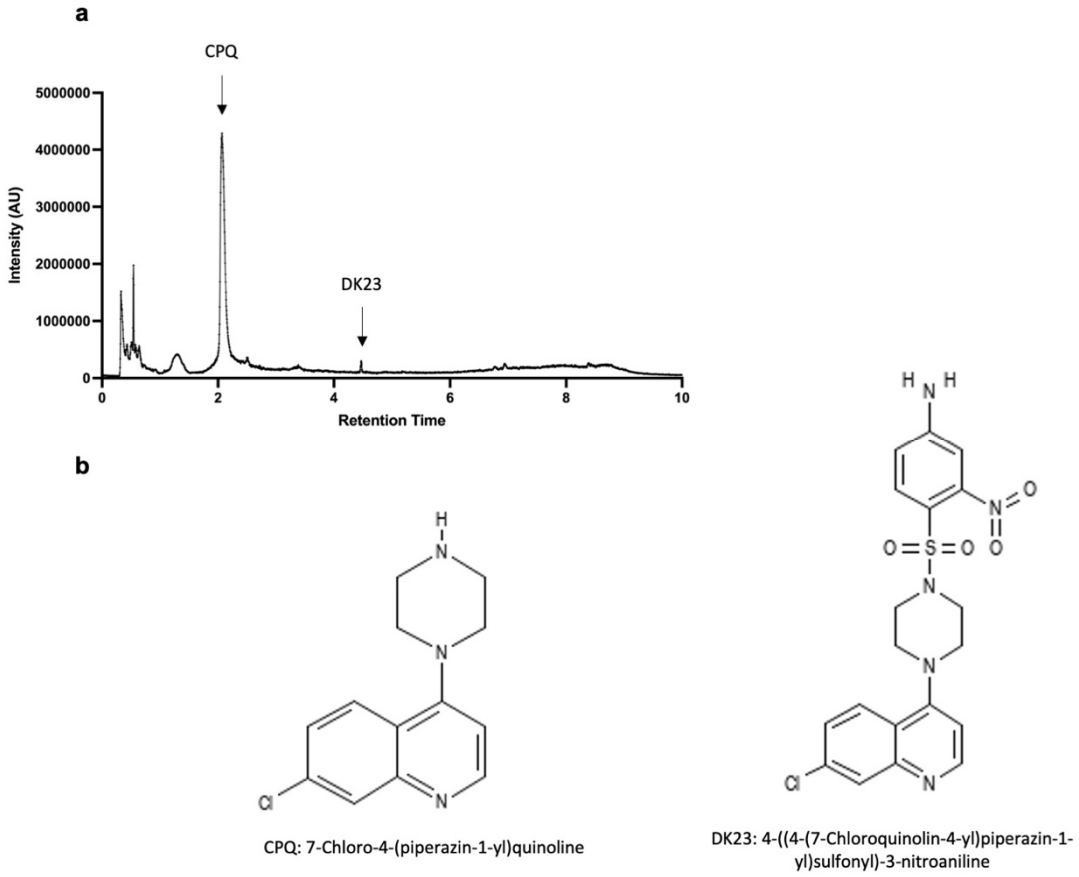

Figure 3.1. The identification of two major VR23 metabolites. (**a**) Whole cell extracts of SW982 cells treated with VR23 (10 μM) for 6 h were dissolved in a 50:50 mix of H2O and Acetonitrile (ACN) and injected onto a reverse phase ACQUITY UPLC BEH C18 10 cm column and eluted using a ballistic gradient from 70:30 H2O:ACN to 20:80 H2O:ACN over the course of 10 minutes. Detection of analytes was carried out by electrospray ionization mass spectrometry. (**b**) DK23 and CPQ were detected by comparison with commercially prepared known mass standards (TRC, Toronto, Canada).

DK23 has active anti-inflammatory properties

Having established that the two major metabolites of VR23 are CPQ and DK23, we determined if either or both of them possess anti-inflammatory activities by measuring the levels of pro-inflammatory cytokines as described previously [216].

We found that CPQ at its IC_{50} concentration of cell growth did not decrease the level of IL-6 in the IL-1β-stimulated cells (Fig. 3.2), indicating that CPQ is not an active VR23 metabolite against inflammation while, as expected, VR23 was highly active (Fig. 3.2). In contrast to CPQ, DK23 at its IC_{50} concentration significantly reduced the level of IL-6 in the stimulated cells. This data demonstrates that the major mechanism of VR23-mediated anti-inflammatory response is through its metabolite DK23.

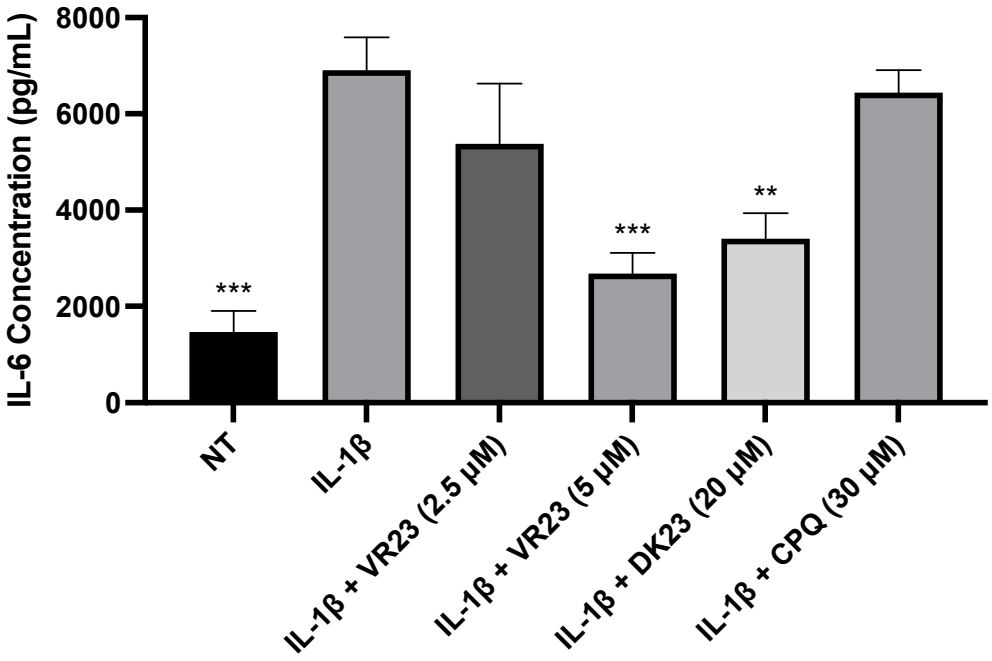

Figure 3.2. The VR23 metabolite, DK23, down-regulates the pro-inflammatory cytokine, IL-6. Shown are the levels of SW982 with IL-1β (10 ng/mL) for 6 h in the absence (control) or presence of CPQ or DK23. For comparison, VR23 at two concentrations was also included. The comparison between groups was made using a one-way ANOVA. The p values were determined by a Dunnett test, and the values presented are mean ± SEM (n = 3). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, which denote significant differences from the IL-1β–only group.

DK23 down-regulates IL-6 and MCP-1 in human primary synovial cells

As demonstrated in Chapter 2, VR23 can effectively down-regulate the IL-6-STAT3 inflammatory pathway in HFLS cells from RA patients. DK23 was then analyzed to determine if it can also downregulate inflammatory responses in primary synoviocytes. DK23, at its IC_{50} concentration shows a trend of down-regulation of the level of IL-6 in cells stimulated with IL-1β by approximately 50%, in comparison to the IL-1β only treated sample (Fig. 3.3a). Interestingly, at 2 times the IC_{50} concentration, VR23 was more effective than DK23 in reducing the level of IL-6 (Figure 3.3a).

In addition to reducing the level of IL-6, DK23 also demonstrated a downregulation trend of the MCP-1 expression level. At the IC_{50} concentrations, VR23 was more effective than DK23 by approximately 10% (50% *vs* 60%) in downregulating the level of MCP-1, while HCQ was similar to that of DK23 (Fig. 3.3c). Similar trends of reducing the levels of IL-6 and MCP-1 by VR23, DK23, and HCQ were also found in human primary synovial cells from a healthy donor (Fig. 3.3b,d). These data indicate that: (1) VR23 is also similarly metabolized in primary synoviocytes to produce DK23, thus VR23 can be a prodrug in human body; and (2) DK23 can be an effective drug to treat RA.

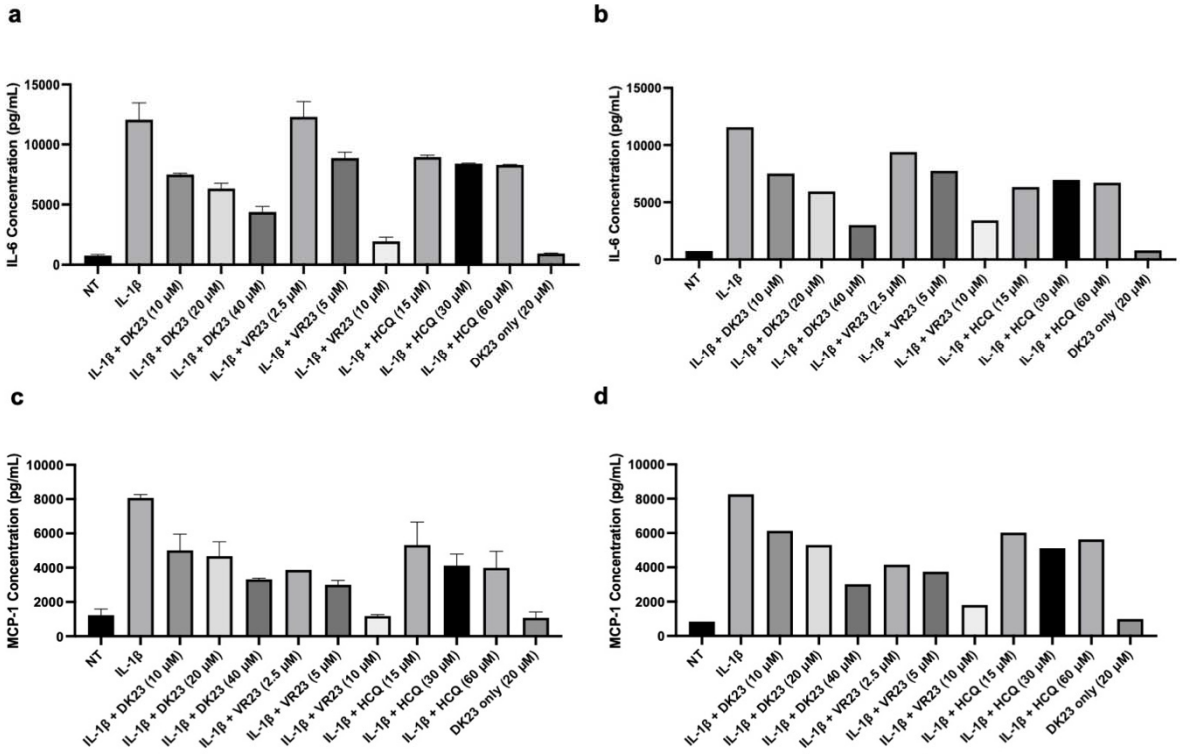

Figure 3.3 DK23, a major metabolite of VR23, down-regulates IL-6 and MCP-1 in human synovial cells. (**a** and **b**) The levels of IL-6 in the supernatant of HFLS-RA (**a**) or HFLS-N (**b**) cells are shown at 6 h post-stimulation with IL-1β in the absence or presence of various doses of either DK23, VR23, or HCQ. (**c** and **d**) The levels of MCP-1 in the supernatant of HFLS-RA (**c**) and HFLS-N (**d**) cells are shown at 6 h post-stimulation with IL-1β in the absence or presence of various doses of either DK23, VR23, or HCQ. The values presented in (**a**) and (**c**) are mean ± SEM (n = 2), while (**b**) and (**d**) are n=1.

DK23 downregulates the STAT3 pathway through decreasing phosphorylation on tyrosine 705 in the IL-6-stimulated primary human synovial cells from RA patients

I showed previously that VR23 prevents the phosphorylation of STAT3 on the tyrosine 705 residue, by which it prevents/reduces inflammatory response. Therefore, I examined if DK23 can also downregulate the level of STAT3 phosphorylation on tyrosine 705. To mimic chronic inflammation in a joint, HFLS cells were stimulated with a combination of IL6 and sIL-6R. Data from HFLS-RA showed that DK23 (~50%) demonstrated a trend of down-regulation of the level of p-STAT3 (Y705), to a similar extent to VR23 (Fig. 3.4a). The trend was similar in HFLS from a healthy donor; however, the degree of the decrease was generally smaller, compared to the decrease in HFLS-RA. Interestingly, HCQ was either not effective or slightly increased the level of p-STAT3 (Y705) (Fig. 3.4).

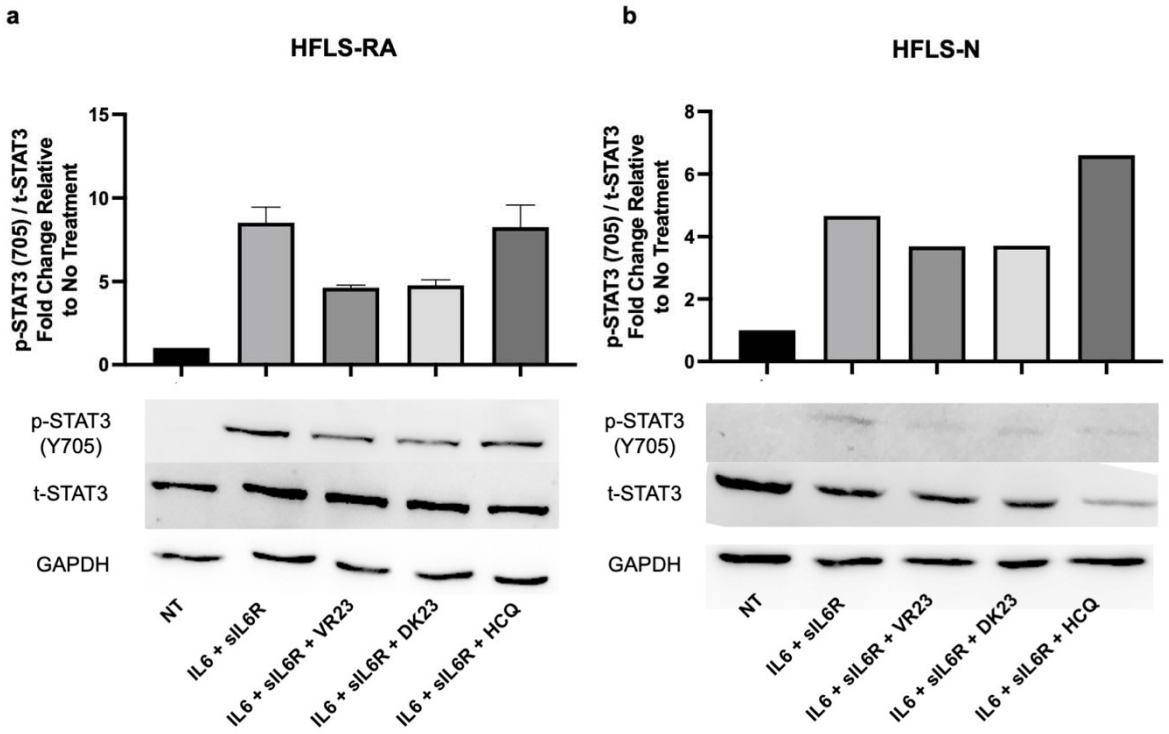

Figure 3.4. Both VR23 and DK23 downregulate the STAT3 pathway through decreasing phosphorylation on tyrosine 705 in the IL-6-stimulated primary human synovial cells from RA patients. Primary HFLS from RA patients (**a**) or healthy donors (**b**) were treated with VR23 (5 μM), DK23 (20 μM), or HCQ (30 μM) for 1 h, stimulated with IL-6 (50 ng/mL) for 15 minutes, and then collected to analyze the level of p-STAT3 (Y705) and total STAT3 (t-STAT3) proteins. GAPDH was used as a loading control. Fold change is displayed in relation to the no-treatment control. The values presented are mean ± SEM (n = 2).

DK23 disrupts lysosomal acidity

We previously demonstrated that VR23 relatively quickly elevates lysosomal pH, which may be relevant to the downregulation of the IL-6/STAT3 inflammatory pathway (Chapter 2). Since DK23 is the major active metabolite of VR23, it may also decrease the level of p-STAT3 (Y705). This possibility was examined using SW982 human synovial cells. Cells were treated for 6 h with either VR23, DK23, HCQ, or BAF, a V-ATPase inhibitor that increases lysosomal pH. The resultant data show that VR23 and DK23 did not increase the fluorescent punctae of the acidic organelles to a similar extent as BAF, indicating both VR23 and DK23 increase the lysosomal pH within 6 h. In contrast, HCQ demonstrated a trend of up-regulating acidic organelles (decreasing pH) under these conditions. Therefore, this data suggests that the elevation of the lysosomal pH by VR23 is through its metabolite DK23.

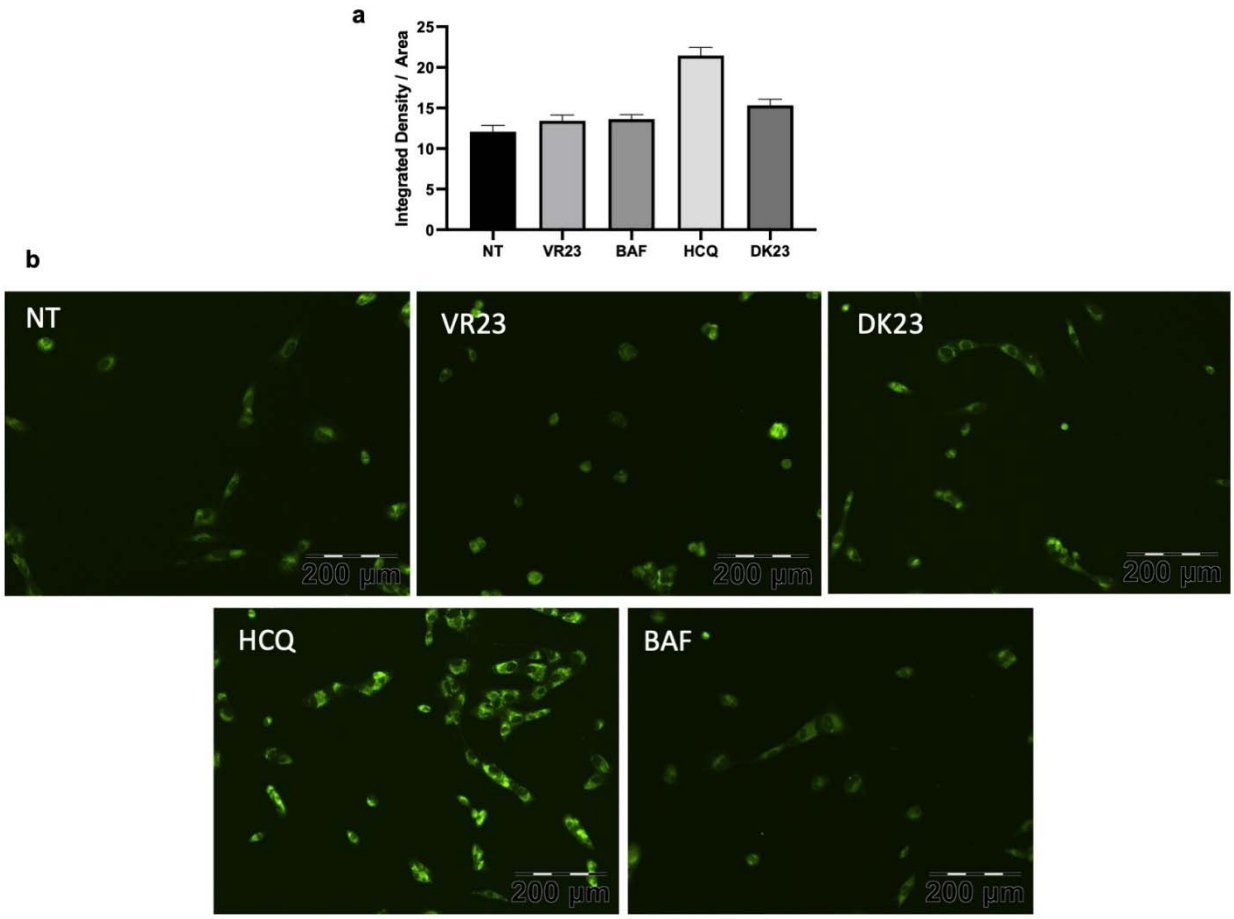

Figure 3.5. DK23 elevates lysosomal pH. SW982 cells were treated with VR23, DK23, HCQ, or BAF at their respective IC_{50} values for 6 h, followed by incubation with lysosensor for 30 minutes prior to imaging. (**a**) Integrated density of cells in the microscopic images shown in panel **b**, normalized to the area of the cells and to background. The values presented are mean ± SEM (n = 2).

Discussion

I have previously documented the anti-inflammatory properties of VR23 through the down-regulation of pro-inflammatory cytokines in acute and chronic inflammatory cell models [214]. Specifically, I have found the ability of VR23 to regulate IL-6, likely through preventing the phosphorylation of STAT3 (Chapter 2). Here, we report that VR23 is rapidly metabolized in human cells into two metabolites, CPQ and DK23. Drugs similar to VR23, such as anti-cancer agents are often processed by drug-metabolizing enzymes such as glutathione S-transferase, aryl sulfatase, and UDP-glucoronyl transferase into less toxic or inactive metabolites [250]. In ideal situations, upon being processed, there would be a delicate balance of detoxification and activation. Upon being taken up by a cell, VR23 is metabolized into the relatively harmless CPQ, which appears to be the most abundant metabolite of VR23. The CPQ structure is the quinoline portion of VR23's main scaffold, indicating it may be a by-product of VR23 being processed by the cell. DK23, however while not present in the highest quantity, has the identical chemical structure with VR23, except that one of the two nitro groups is replaced with an -NH2 group.

Upon examining the anti-inflammatory activity of the two metabolites, CPQ was not active but DK23 has similar potency as VR23. My data clearly shows that DK23 can down-regulate pro-inflammatory cytokine at a similar efficacy as VR23 at their respective IC_{50} concentrations. CPQ did not show any activity in mediating pro-inflammatory cytokines. The cleavage of VR23 is likely through sulfonamide cleavage by glutathione-S-transferase (GST), as prior studies have found that similar drugs such as protease inhibitors are processed in this manner [251]. In other DMARDs, some of the metabolites are active and are able to help mitigate inflammation, and others are simply metabolic by-products, perhaps as part of the detoxification process.

A promising aspect of VR23's anti-inflammatory properties is its ability to down-regulate the pro-inflammatory mediators in both acute and chronic inflammatory settings. MCP-1 and IL-6 are found in high levels in synovial fluid and in the serum of patients with RA, implicating their importance in the progression of this disease [100, 252]. VR23 has shown great potential as an anti-rheumatic agent, through its ability to mitigate IL-6 and MCP-1 secreted by HFLS cells from RA patients [216]. Data from our cytokine assays clearly demonstrate that DK23 is

effective at down-regulating IL-6 and MCP-1 in RA HFLS cells, at a similar potency as VR23. Interestingly, at a higher concentration, VR23 appears to be more effective at reducing the cytokine concentrations than DK23. This difference could be attributed to the toxicity of VR23 as an anti-cancer agent, while DK23 as a metabolite is less toxic. Typically, nitro-group containing drugs have higher toxicity profiles, however the metabolite DK23 has one less nitro group than VR23, indicating it may be less toxic and a preferable option for anti-inflammatory treatment [253].

In response to IL-6 stimulation, DK23 was effective in preventing STAT3 phosphorylation on tyrosine 705 to a similar extent as VR23, although the former was used at a higher concentration than the latter. This confirms that the active metabolite, DK23 is effective in mitigating inflammation through the IL6-STAT3 pathway. To further this, I confirmed that DK23 also demonstrated the ability to increase lysosomal pH at a similar capacity to VR23, and BAF. Consistent with my prior studies, HCQ did not demonstrate the ability to increase the lysosomal pH under the same experimental conditions.

Overall, I have demonstrated here that upon being metabolized by a cell, VR23 is converted into CPQ and DK23, the latter is structurally very close to VR23. While CPQ is inactive, DK23 showed quite potent anti-inflammatory properties of VR23. DK23 showed quite potent anti-inflammatory activity in HFLS-RA cells in reducing IL-6, MCP-1 and p-STAT3. VR23, as a novel anti-inflammatory drug may act as a pro-drug by converting into DK23, which actually possesses anti-inflammatory activity.

5.0 General Discussion

Throughout this study, I studied the novel anti-cancer agent, VR23 for its potential as an anti-inflammatory agent. Further, I investigated the mechanism by which VR23 acts to down-regulate inflammatory markers.

In Chapter 1, I investigated the ability of VR23 to down-regulate pro-inflammatory markers in an acute inflammatory cell model. I observed previously that the level of macrophages in a mouse spleen treated with VR23 was much lower than those treated with other compounds such as paclitaxel. From this data and the fact that VR23 was created as a hybrid molecule of HCQ, we thought that VR23 would have anti-inflammatory capabilities. I found that in an acute inflammatory cell model VR23 can effectively down-regulate pro-inflammatory cytokines such as IL-6, TNF-α, IL-1β, and IL-8. In addition, VR23 presented potent anti-inflammatory capabilities in reducing IL-6 and MCP-1 in chronic inflammatory cell models, including HFLS cells from RA patients. Lastly, VR23 showed effectiveness in an acute lung injury mouse model by reducing cytokine secretion, cell recruitment, and myeloperoxidase activity. In Chapter 1, I found that VR23 indeed possesses quite potent anti-inflammatory property. It also furthered our understanding of VR23 and its effectiveness in specifically reducing IL-6 and MCP-1 in both acute and chronic inflammatory settings. This study showed the potential for VR23 to be used as a novel anti-inflammatory drug, in addition to increasing its effectiveness as an anti-cancer drug.

In Chapter 2, I investigated the mechanism by which VR23 is able to reduce inflammation. In Chapter 1, I showed that VR23 effectively down-regulates the levels of IL-6 and its down-stream gene expression. This Chapter focuses on unraveling the potential for VR23 to disrupt the IL-6-STAT3 pathway as a mechanism for how it may prevent inflammation. With a focus on IL-6 related pathways, I examined the effects of VR23 on LPS induced p-STAT3, CREB, and SOCS3 and I found that VR23 effectively down-regulates p-STAT3. In the IL-6 induced inflammatory model, I further corroborated this by demonstrating VR23's potency at reducing p-STAT (Y705), while not affecting upstream markers of the IL6-STAT3 pathway. Hydroxychloroquine, a well-known DMARD being frequently used at clinics, showed no effectiveness in mitigating p-STAT3, while VR23 demonstrated the same effectiveness as an IL-

6R specific inhibitor, LMT-28. Further, the gene expression of down-stream IL-6-STAT3 pathway proteins, MCP-1 and c-myc were also down-regulated by VR23. To determine if VR23 could affect the IL-6-STAT3 pathway in a chronic RA model, I examined its capabilities in primary HFLS from RA patients and healthy donors. VR23 demonstrated effectiveness in preventing the phosphorylation of STAT3 in HFLS cells from RA patients, while the clinically used DMARD, HCQ, was not effective. Systemic inflammation often involves the production and circulation of CRP [254], which is induced by IL-6 stimulation in hepatocytes [255]. In this model I showed that VR23 has the potential to prevent the phosphorylation of STAT3, while reducing the CRP cytokine levels. Hydroxychloroquine is known for its ability to increase the pH of acidic organelles, which in part is the mechanism that helps to mitigate inflammation. VR23 was tested in parallel with HCQ to determine their abilities to inhibit phagocytosis and increase the pH of acidic organelles. Interestingly, we found that VR23 was very effective at inhibiting phagocytosis in comparison to HCQ. In addition, VR23 was able to easily increase the pH of acidic organelles, while HCQ actually decreased the lysosomal pH. I found that VR23 has the ability to mitigate acidic organelle's pH in a relatively shorter time frame. This is a contrast with HCQ which requires quite a long time to show its effects in elevating lysosomal pH.

This study unraveled the effects of VR23 on IL-6, by demonstrating its potency in preventing the phosphorylation of STAT3 (Y705) in monocytes, HFLS cells, and hepatocytes. It also further showed the ability of VR23 to increase the pH of acidic organelles which may be attributed to its effects on the phosphorylation of STAT3. In the future, VR23 could be analyzed in a collagen-induced arthritis mouse model to further understand its effects on mitigating p-STAT3 and IL-6 in sera and synovial tissue.

In Chapter 3, I investigated VR23 treated cells to determine if cells metabolize it and whether the metabolites are active. An LCMS-based study revealed that VR23 is metabolized into two major products, CPQ and DK23, in human cells. I confirmed the identities of the two metabolites by synthesizing CPQ and DK23 and analyzing them by LCMS. I then examined them for their anti-inflammatory activities. DK23 showed anti-inflammatory activity, of which potency was comparable to that of VR23 at their respective IC_{50} concentrations. In contrast, CPQ was inactive in terms of mitigating inflammation. Specifically, DK23, as an analog of VR23,

showed similar anti-inflammatory effects as VR23 in reducing MCP-1, IL-6, p-STAT3 (Y705) in HFLS-RA cells. These findings should be corroborated in the future with more RA patient samples, as limited samples were available. Perhaps importantly, the VR23 metabolism study led us to the discovery of the DK23 novel anti-inflammatory agent. Thus, it is warranted to further characterize DK23 including *in vivo* toxicology, solubility and the route of administration, etc. in order to develop it as a potential anti-inflammatory and anti-rheumatic drug.

Overall, my study has demonstrated great potential for VR23 as a novel anti-inflammatory agent. VR23 has the potential to be used in both acute and chronic settings of inflammation, specifically for RA. VR23 is superior as an anti-inflammatory to the currently available DMARDs, as its mechanism is more specific and it has a low toxicity profile. Further, in comparison to hydroxychloroquine, VR23 appears more effective as it can reduce inflammatory markers more effectively and in a timely manner. VR23 works to prevent the phosphorylation of STAT3 and its down-stream genes, leading to the reduction of pro-inflammatory cytokines such as IL-6 and MCP-1, which play a critical role in RA disease progression. This allows VR23 to have similar specificity to biologics, with less systemic toxic side effects. DK23, the major active metabolite of VR23, demonstrates similar anti-inflammatory effects as VR23, which functions through the regulation of the IL6-STAT3 pathway. The validation of DK23's activity furthers our understanding of VR23 as a novel anti-inflammatory agent. These findings also expand the potential for DK23 in its ability to be used on its own as a novel anti-inflammatory compound for chronic inflammatory conditions. VR23 and its metabolite, DK23, have great potential as novel compounds for the treatment of chronic inflammatory conditions such as RA by being effective, selective, and low in toxicity.

6.0 References

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7.0 Appendix

Review

The Potential of Combining Tubulin-targeting Anticancer Therapeutics and Immune Therapy

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Abstract: Cancer immune therapy has recently shown tremendous promise to combat many different cancers. The microtubule is a well-defined and very effective cancer therapeutic target. Interestingly, several lines of evidence now suggest that microtubules are intimately connected to the body's immune responses. This raises the possibility that the combination of microtubule inhibitors and immune therapy can be a highly effective option for cancer treatments. However, our understanding on this potentially important aspect is still very limited, due in part to the multifaceted nature of microtubule functions. Microtubules are not only involved in maintaining cell morphology, but also a variety of cellular processes, including the movement of secretory vesicles and organelles, intracellular macromolecular assembly, signaling pathways, and cell division. Microtubule inhibitors may be subdivided into two classes: Anti-depolymerization agents such as the taxane family, and anti-polymerization agents such as colchicine and vinka alkaloids. These two different classes may have different effects on immune cell subtypes. Anti-depolymerization agents can not only induce NK cells, but also appear to inhibit T regulatory (Treg) cells. However, different inhibitors may have different functions even among the same class. For example, the docetaxel anti-depolymerization agent up-regulates cytotoxic T cells,

while paclitaxel down-regulates them. Certain anti-polymerization agents such as colchicine appear to down-regulate most immune cell types, while inducing dendritic cell maturation and increasing M1 macrophage population. In contrast, the vinblastine anti-polymerization agent activates many of these cell types, albeit down-regulating Treg cells. In this review, we focus on the various effects of tubulin inhibitors on the activities of the body's immune system, in the hope of paving the way to develop an effective cancer therapy by combining tubulin-targeting anticancer agents and immune therapy.

Keywords: microtubule inhibitors; immune therapy; chemotherapeutics; cell cycle dysregulation; immune cells; cytokines

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1.A Brief Overview of Microtubules in the Context of Mammalian Cell Cycle

The cell division cycle is a series of events leading to the formation of two mitotic daughter cells with a complete set of the entire genome for each cell [1]. The cell cycle comprises two compartments: Interphase and mitotic (M) phases. The interphase, occupying approximately 22–23 h of the typical 24 h cell cycle, can be further subdivided into G1 (gap1), S (synthesis), and G2 (gap 2) phases [1]. Large part of the cellular contents are duplicated during G1; the complete and accurate duplication of the entire genome occur during S phase; and the duplicated genome undergoes a "verification" process in G2 [1]. When cells are not in an active proliferation state, they may be arrested at a resting stage called G0 phase for a prolonged period. M phase typically lasts only 1–2 h. Like interphase, M phase can also be subdivided into various stages. The first stage, termed prophase in mammalian cells, involves chromosome condensation, and nuclear membrane breakdown, followed by duplication and moving of the centrosome toward two poles, and mitotic spindle formation [2]. The microtubule plays an essential role in this stage, particularly for centrosome movement and spindle formation. The attachment of the microtubules at opposite spindle poles allows for the chromosomes to be pulled towards the ends at the subsequent stages. During the second stage, termed metaphase in mammalian cells, chromosomes are further condensed and aligned at the cell's equator [2]. One of the most important cell division controls called spindle checkpoint operates during this cell cycle compartment. The third stage, anaphase, discerns the separation of the sister chromatids, which is the result of the degradation of securin

by separase and the shortening of microtubules [2]. The chromosomes migrate towards the spindle poles following their separation [2]. The final stage of mitosis, telophase, occurs when the chromosomes reach their respective poles, followed by cytokinesis. The optimal microtubule dynamics are critical for the smooth cell division process [3].

Microtubules are composed of heterodimeric chains of α-tubulin and β-tubulin molecules. There is a third subtype termed γ-tubulin in eukaryotes, which is needed for the nucleation of the α/β-tubulin polymerization [4]. The various interactions between the tubulin dimer in the microtubules are responsible for maintaining its tubular form as well as trafficking proteins and organelles [4]. While the microtubule is a key regulator of cell division, its dysregulation may contribute to the development of tumorous cells, as evidenced by the fact that the majority of tumor cells display aneuploidy [5]. Furthermore, tubulin mutations can also contribute to the development of chemo-resistance and tumor propagation through altered responses to cell microenvironment [5]. Some of these mutations have been identified through notable differences between tubulin isomers, tubulin post-translational modifications, and differences in the tubulin associated molecular patterns [5]. While a specific oncogenic tubulin isotype is yet to be discovered, many studies have shown that oncogenic pathways such as the AKT and ERK pathways function through the microtubule [6].

2. The Immune System as the Body's Security Guard

During oncogenic activation and the subsequent cellular proliferation stage, cancerous cells may be recognized and eliminated through the body's immune system [7]. As more mutations accumulate, pathogenic cells including cancer cells may present more cell surface antigens which in turn activate different components of the immune system, ultimately leading to their demise by the immune system [8]. Unfortunately, some cancer cells may develop special mechanisms to exploit and overcome their elimination by the immune system [8]. The good news is that the immune system can activate different subsets of mediators, which are dependent upon the presentation of a primary tumor (initial tumor) or a secondary tumor (a new tumor derived from the primary tumor) [7]. Some of these key mediators include M1 macrophages, natural killer cells (NK), neutrophils, T cells, B cells, and dendritic cells, among others [9]. This aspect will be discussed in more detail below.

3. Tubulin Inhibitors Effectively Target Cycling Cancer Cells

While the immune system alone may be able to eliminate highly proliferative metastatic tumor cells, this process can be more effectively achieved when combined with other therapies. There are many different anticancer agents targeting various components of cell regulation, especially the mitotic stage of tumor cell proliferation. Although the microtubule is an effective target at different stages of cell regulation, many microtubule inhibitors are especially effective at the mitotic stage. Agents targeting tubulin may be classified into two major classes: Anti-depolymerization and anti-polymerization of the microtubule cytoskeleton (Figure 1). Among microtubule depolymerization inhibitors, taxanes are arguably the most effective compounds, and have been used effectively for the treatments of several different cancers [10]. The binding of a taxane compound to its binding-site inhibits the depolymerization of the microtubules, leading to mitotic arrest and eventual tumor cell death [11]. Paclitaxel, a first generation taxane, was initially isolated from the tree *brevifoliaI* and utilized to treat breast cancer [11]. For clinical administration of paclitaxel, nab-paclitaxel (nanoparticle albumin-bound paclitaxel) allows for a higher solubility of the drug, enhancing its delivery to patients [12]. Nab-paclitaxel also decreases the toxicity associated with paclitaxel delivery to patients [12]. Due to its high demand and scarcity of the natural sources, its semi-synthetic version docetaxel was developed [11]. Studies with tumor cell lines showed that docetaxel is a 1.3–12 fold more effective than paclitaxel [13,14]. Docetaxel, unlike paclitaxel, displays linear pharmacokinetics and is thus retained intracellularly for a longer period of time [15]. Compounds binding to the taxane-binding site may also inhibit the Bcl-2 gene activation (through phosphorylation), thus promoting apoptosis, in addition to stabilizing microtubules (Table 1) [16].

The second class of microtubule inhibitors works by inhibiting microtubule polymerization, which may be further divided into two subclasses based on their targets: The vinca-binding domain or the colchicine-binding domain. Vinca alkaloids, the prototype of the former subgroup, are originally from the periwinkle plant, *Catharanthus roseus,* and are often used to treat a variety of different neoplasms [17]. Contrary to taxanes, vinca alkaloids bind directly to the tubulin dimer, thus disrupting microtubule functions (Table 1) (Figure 1) [17]. As a result of the disruption, the mitotic spindle becomes defective, leading to a prolonged metaphase arrest [17]. Another difference is that vinca alkaloids bind rapidly to the tubulin in a reversible manner, while taxanes and colchicine site-binding compounds do not [18].

Colchicine site-binding compounds are also important microtubule polymerization inhibitor. Colchicine alkaloids, originally derived from plant *Autumn crocus,* have been well-documented for their use for the treatments of gout, inflammation, and possibly cancer [19]. Similarly to vinca alkaloids, colchicine compounds bind to the colchicine-binding site on the β-tubulin, inhibiting microtubule polymerization and leading to a prolonged metaphase arrest (Table 1) [19]. Unlike vinca alkaloids, however, colchicine binds to the tubulin in a poorly reversible manner, leading to the prevention of microtubule polymer elongation [19]. Microtubule growth arrest or microtubule depolymerization are dose dependent with a higher dose causing the latter response [19].

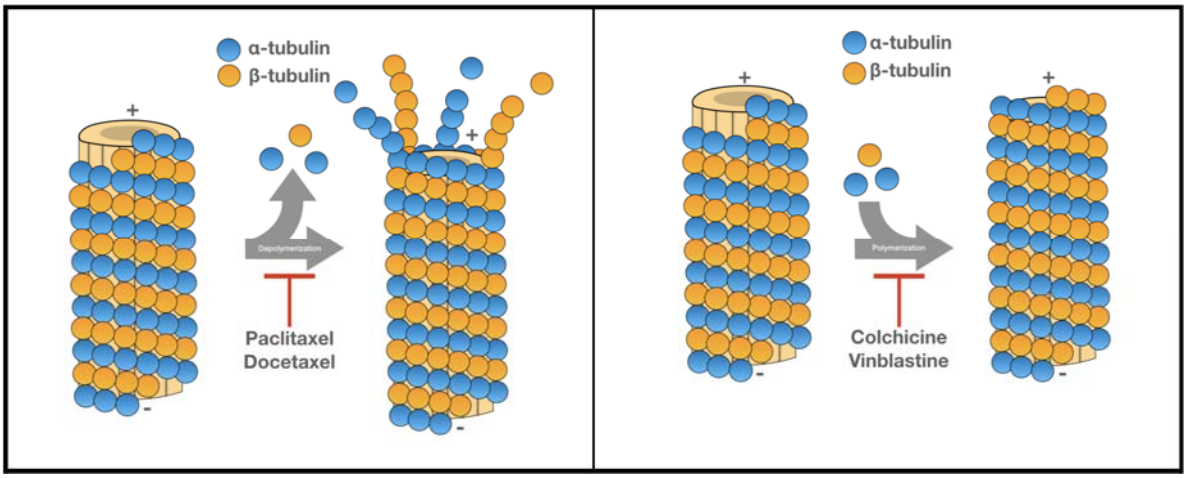

Figure 1. Demonstrates how the tubulin inhibitors affect the microtubules by preventing depolymerization or polymerization. Panel left illustrates the effects of paclitaxel and docetaxel (depolymerization inhibitors), while panel right illustrates the effects of colchicine and vinblastine (polymerization inhibitors).

Having briefly described the immune system and different roles of microtubule inhibitors, the current review aims to provide insights into microtubule inhibitors in the context of the body's immune responses. We here describe how different "classes" of tubulin-targeting agents up-regulate or down-regulate the immunomodulatory activity of T cells, NK cells, monocytes, and dendritic cells. There is an excellent possibility that the chemo-immuno combinational therapy can substantially improve the outcome of cancer treatments. To achieve this goal, it will be imperative to understand if and how anticancer chemotherapeutic agents affect the regulation of the body's immune responses.

Table 1. Summary of well-known tubulin inhibitors.

Microtubule Inhibitors	Binding Domains	Cancer Treatments	Mode of Action	References
Paclitaxel (nab-paclitaxel)	Taxane-binding	Breast, ovarian, prostate, lung	Anti-microtubule depolymerization leading to mitotic arrest	[12,20]
Docetaxel	Taxane-binding	Breast, non-small cell lung, androgen-independent metastatic prostate cancer	Anti-microtubule depolymerization, and attenuation of bcl-2 and bcl-xL gene expression	[21,22]
Colchicine *	Colchicine-binding	Hepatocellular & prostate cancers	Anti-microtubule polymerization. Cell cycle arrest in metaphase	[19,23–25]
Vinblastine	Vinca-binding	Testicular, Hodgkins and non-Hodgkins lymphoma, breast, & germ cell cancers.	Induces wedge at tubulin interface causing tubulin self-association into spiral aggregates. Anti-microtubule polymerization, & cell cycle arrest in metaphase.	[17,26]

* Colchicine is often administered for the treatment of gout as it was FDA approved for this condition in 2009. While colchicine has not yet been approved for cancer treatment, it was shown to decrease cancer incidence in male gout patients [25].

4. Regulation of T Cells

T cells are the integral part of the immune system as they are responsible for cell-mediated immune reactions [27]. Naïve T cells are immunologically not active and can only differentiate into their effector states (CD4+ or CD8+) upon the recognition of foreign antigens presented by antigen presenting cells such as dendritic cells, macrophages, and B cells [27]. T cells can take on an activated phenotype of either CD4+ helper function or CD8+ cytotoxic capabilities [28].

CD8+ cytotoxic T cells are essential for providing protection against pathogens, as they can directly kill and remove pathogen-infected cells [27]. To kill an infected cell, a cytotoxic T cell may release perforin to create pores in the plasma membrane of the targeted cell, allowing proteases to enter the cell and promote its death [27]. Cytotoxic T cells can also induce apoptotic cell-killing by activating the caspase cascade in the target cell [27]. CD4+ T cells are involved in the adaptive immune response by becoming T helper cells upon stimulation by an APC (antigen presenting cell). The activated T helper cells then can stimulate B cells or cytotoxic T cells, while secreting cytokines and chemokines to activate neighboring cells. T helper cells are further divided into Th1 and Th2 cells according to the cytokines they produce [28]. Th1 cells secrete TNF-α and IFN-γ to activate macrophages and cytotoxic T cells, which promotes the killing of intracellular pathogens [27]. Th2 cells secrete several different cytokines including IL-4, IL-5, IL-10 and IL-13 to help defend the body from extracellular pathogens [27]. The main effect of these interleukin cytokines is to stimulate B cells to create antibodies [27]. The antibodies would then bind to innate immune cells (i.e., mast cells, basophils, and eosinophils) to induce local mediators to fight off infection [27]. In addition to Th1 and Th2 subsets, there are other T helper cells that play an important role for immune responses, including Treg, Tfh, and Th17 cell types [28]. Treg cells may suppress the immune response by down-regulating the proliferation of T cells and their cytokine productions, which is important for the body to prevent the induction of autoimmune responses [29]. Treg cells are typically increased in the tumor microenvironment to inhibit the cytotoxic T cells from killing cancer cells, a typical mechanism of cancer cells evading the immune surveillance [30]. In general, T cells work to protect our bodies from infections and to eliminate foreign invaders by facilitating an effective immune response.

5. Responses of T Cells to Microtubule Inhibitors

The treatment of colchicine on healthy individuals results in the reduction of both T helper and cytotoxic T cell populations [31]. However, the exact mechanism of how this global decrease

in the overall T cell population by colchicine is unknown, although the down-regulation of cell division cycle could certainly be one reason.

The effects of paclitaxel on T cells have been studied more in detail by several groups. For example, Vicari and colleagues demonstrated that paclitaxel substantially decreases the numbers of Treg cell numbers in the spleen of both normal and tumor-bearing mice [32]. Further investigation revealed that the level of FOXP3 expression was substantially decreased in Treg cells in response to paclitaxel, indicating a decrease in the inhibitory capacity of Treg cells [32]. As a result, Treg cell-mediated T cell proliferation was severely impaired [32]. Typically, the down-regulation of Treg cells increases the CD8+ T effector cell population, due to a decrease in the Treg-mediated suppression of CD8+ cells [32]. Surprisingly, however, paclitaxel's ability to decrease Treg cells did not result in an up-regulation of CD8+ T cell activities [32]. Paclitaxel actually down-regulates both Treg and CD8+ T cell subtypes, probably due to the toxic effects of paclitaxel on the cycling cells [32]. This indicates that paclitaxel does not promote immune responses, but suppresses overall T cell population and thus their activities.

Mullins and colleagues investigated in a murine model whether paclitaxel-mediated immunosuppression on T cells can be reversed by IL-12 [33]. The authors found that the administration of exogenous IL-12 can not only overcome the paclitaxel-mediated suppression of CD4+ T cells, but actually promote cell proliferation [33]. This result suggests that, while paclitaxel alone reduces the T cell population, the combination of paclitaxel with IL-12 can have positive effects on T cell activities [33].

Li and colleagues (2014) found that docetaxel decreases Treg cells in patients with non-small cell lung cancer [34]. Similar to this study, Turk et al. (2004) found that docetaxel treatment resulted in the decrease of Treg cells in a mouse model, leading to an increase in cytotoxic T cells [30]. Thus, docetaxel has the potential of increasing its anti-tumor activity by promoting T cell-mediated tumor cell killing, in addition to its direct tumor cytotoxicity.

Similarly, vinblastine suppresses tumor-induced Treg cells [35]. In this case, the killing of the tumor cells appears to work by increasing the CD8+ T cell population, as vinblastine was not effective when CD8+ T cells were depleted in the host animals [35]. Therefore, vinblastine has the ability to selectively eliminate Treg cells, while not reducing the CD8+ T cell population. Thus, vinblastine can enhance the killing of cancerous cells by immune system [35].

Taken together, certain tubulin-targeting anticancer agents have the potential of increasing their anti-tumor activities by down-regulating Treg cells (Figure 2). In most of these cases, the drugs selectively target the regulatory T cells while sparing/increasing the proliferation of cytotoxic T cells, allowing cytotoxic T cells to attack the cancer cells. Unfortunately, paclitaxel, one of the most commonly used tubulin-targeting agents, reduces both the Treg cells and the cytotoxic T cell populations. These aspects should be an important consideration for a combined cancer therapy with anti-microtubule agents and immunogenic therapeutic agents such as checkpoint blockades.

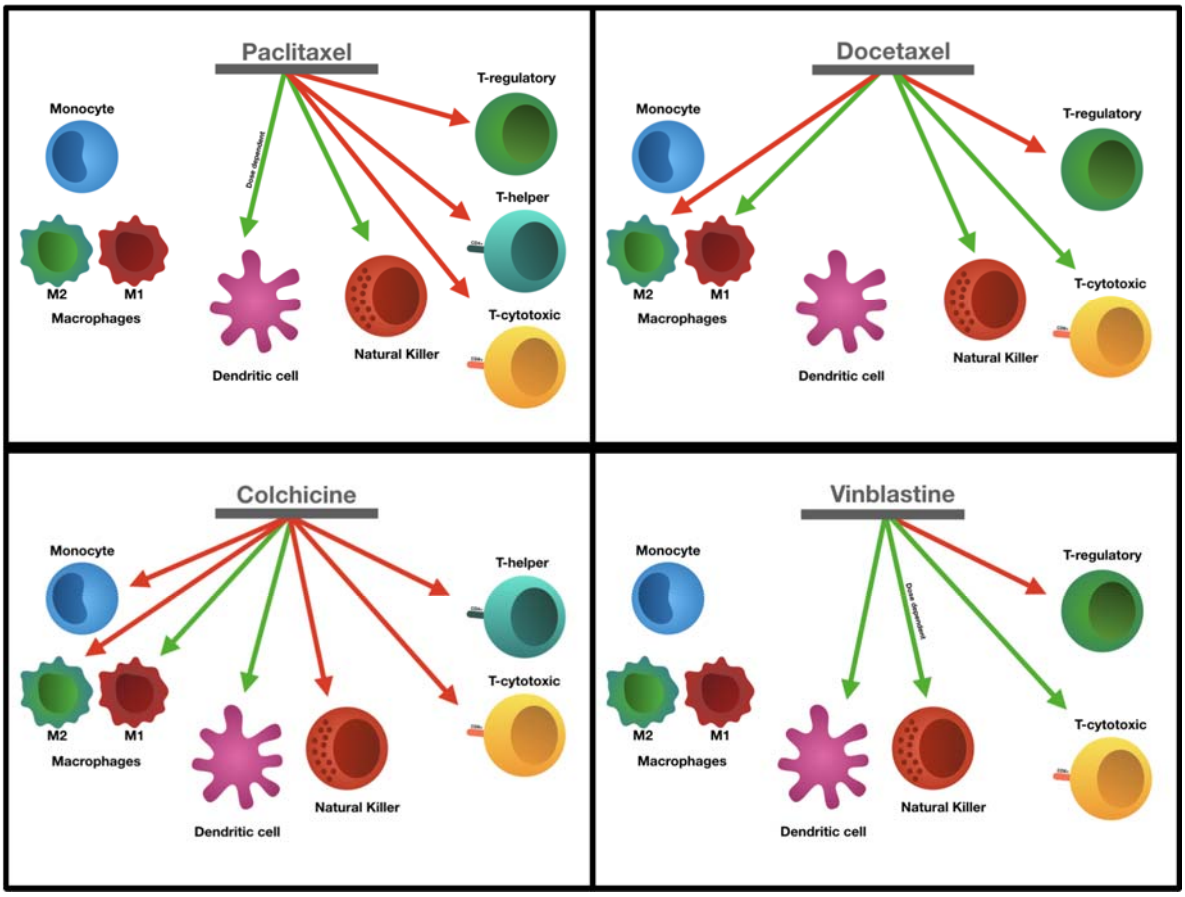

Figure 2. Tubulin inhibitors and their effects on the various immune cell types. Green arrows indicate the ability of the drugs to induce activation and red arrows indicate the inhibition of that immune cell type.

6. Regulation of Monocytes

Monocytes are a type of white blood cells, accounting for approximately 5% of the leukocytes in the body. They are found in circulating blood, bone marrow, and spleen [36,37]. Monocytes have many functions in the body, including maintaining homeostasis and assisting of immune defense and tissue repair [38]. As effector immune cells, monocytes circulate in the blood stream and migrate into specific sites if or when infections happen [36]. One major effector function of monocytes is to secrete inflammatory cytokines to combat foreign invaders/microbial infections [36]. Monocytes are often divided into three types based on the varying levels of CD14 and CD16 surface markers [38]. A typical monocyte has a high expression level of CD14 and absence of CD16, while a non-classical monocyte has a low level of CD14 and no expression of CD16 [38]. The intermediate monocyte is identified by a high level of CD14 with a low level of CD16 [38]. The intermediate monocyte is thought to be a subpopulation of monocytes that act during reparative processes, as they have high levels of surface markers such as CXCR4 and vascular growth factor [39].

Monocytes are also capable of differentiating into dendritic cells or macrophages, especially in response to inflammation [36]. Macrophages are phagocytic cells that can detect and engulf foreign substances, pathogens, and cellular debris via phagocytosis [40]. Macrophages detect pathogens and help activate the adaptive immune system by presenting antigens, inducing the production of inflammatory cytokines [36]. Macrophages increase inflammation and stimulate the immune system. Interestingly, however, macrophages can also be anti-inflammatory, as described below. Macrophages that induce inflammation are referred to as M1 macrophages, while the M2 macrophages decrease inflammation and promote tissue repair [40].

Monocytes and macrophages play an important role in the tumor microenvironment. Upon recruitment to tumors, monocytes may differentiate into tumor associated macrophages, which promote the initiation, progression, and metastasis of tumors [41]. Understanding the mechanism of how monocytes and macrophages behave in response to microtubule inhibitors is critically important, since relevant drugs may enhance or suppress the immune cell-mediated tumor cell killing.

7. Responses of Monocytes and Macrophages to Microtubule Inhibitors

Manie and colleagues (1993) found that microtubule inhibitors promote the secretion of IL-1 by human monocytes [42]. The authors also found that IL-1 expression by colchicine is due to the disruption of the microtubules, leading to the activation of protein kinase A [42]. The protein kinase A pathway is necessary but not sufficient to induce IL-1 production by disrupting the microtubules [42]. Unlike IL-1, the expression of IL-6 and TNF-α cytokines is not stimulated by colchicine [42].

A study carried out with mouse monocytes and macrophages revealed that colchicine could prevent monocyte proliferation and differentiation [43]. The authors found that the level of pro-inflammatory M1 macrophages is up-regulated, while that of anti-inflammatory M2 macrophages is down-regulated in the presence of colchicine [43]. These data demonstrate that colchicine can simultaneously induce pro-inflammatory macrophages and prevents monocyte differentiation [43]. Thus, it is clear that the contradictory effects of colchicine on inflammation and immune system should be investigated further if we want to utilize a colchicine-immune combinational therapy. Similar to colchicine, docetaxel also appears to promote the differentiation of human monocytes into pro-inflammatory M1 type macrophages, while slightly decreasing the anti-inflammatory M2 macrophages [44]. This is a contrast to paclitaxel, which does not alter the differentiation of M1 or M2 macrophages [44]. However, both of them do promote the activation of monocytes and macrophages *in vitro* [44]. Interestingly, the activation of monocytes by docetaxel may be relevant to its induction of IL-8 and IL-1β. The authors noted that paclitaxel can affect the levels of the cytokines only at a high concentration [44].

Taken together, colchicine effectively reduces monocytes, while inducing the pro-inflammatory M1 macrophages. Docetaxel promotes the differentiation of monocytes into M1 macrophages, whiles inducing cytokine secretion in monocytes. Paclitaxel appears to have no effect on the differentiation of monocytes and their cytokine secretion unless administered at a very high dose (Figure 2). Thus, different tubulin-targeting agents show different effects on immune responses.

8. Regulation of NK Cells

Natural killer cells, a specific type of lymphocyte, constitute an important component of anti-tumoral immunity with their ability to secrete various cytokines and chemokines as well as displaying cytotoxic activity [45]. Mature NK cells, which comprise 10–15% of blood

lymphocytes, circulate through the blood stream to find and eliminate harmful foreign bodies [46]. Natural killer cells possess a specific phenotypic trait as they are all present with CD56 antigens on the cell surface [46]. Physiologically, NK cells produce IFN-γ, thus mediating adaptive immune responses as well as regulating antibody-dependent cytotoxicity through CD16 [46]. Natural killer cells also express receptors for MHC I molecules [45]. Importantly, NK cells have the ability to regulate a variety of cell types including dendritic cells, through the secretion of various cytokines [45,47].

Unlike T cells, NK cells do not require any previous stimulation or an adaptive immune response to elicit its killing properties [47]. NK cells possess only limited clonal expansion, which may be useful in limiting the quantity of cytokines being released in circulation [47]. In the tumor microenvironment, however, NK cell (like T cells) are subjected to the down-regulation of their receptor as a mean to circumvent their cytokine release during neoplasm development [47]. Historically, NK cells have been examined to prevent metastases without much success, even at a very high activity. This failure led to the hypothesis that NK cells do not play any major role in defense against tumors that do not circulate through the blood stream [48]. Activated NK cells, found in well-vascularized tumor microenvironments, are dependent upon the stimulation of IL-2 or IL-15 [48]. This stimulation allows NK cells to survive, proliferate, and maintain functional activity. Without continuous stimulation, NK cells may begin to undergo apoptosis by 24 h [48]. Provided there is sufficient IL-mediated stimulation, NK cells can target cancerous cells through the activation of the NKp46 receptor. This allows the induction of IFN-γ, which leads to the expression of fibronectin [49]. Glasner et al. showed that Ncr1-l (mouse equivalent to NKp46) knock-out mice have increased neoplastic potential, although it did not affect primary tumor growth [49].

9. Responses of NK Cells to Microtubule Inhibitors

Kubo et al. showed that clinically relevant concentrations (nanomolar range) of paclitaxel substantially increase the NK cell-mediated cytotoxicity against the BT-474 breast adenocarcinoma cells [50]. The same study also showed that paclitaxel induces the activation of NF-κB while increasing the production of perforin, a key molecule of NK cell-mediated cytotoxicity [50]. Di Modica and colleagues examined the expression of various ligands in the BT-474 and MDA-MB361 breast carcinoma cells treated with 100 nM of docetaxel [51]. In this study,

the authors found substantially increased expression of NKG2D, an important regulator of NK cell activation [51]. The authors also found that there is no phenotypic difference between NK cells in a pre-clinical mouse model before and after docetaxel treatment [51]. In contrast, 10 μg/mL of paclitaxel inhibited cytotoxicity against both NK cell-sensitive (K562 ovarian cells) and NK cell-resistant (OV-2774) cell lines [52].

While certain taxanes enhanced NK cell activity, the same cannot be said about colchicine (see Figure 2). In a study conducted in 2013, Orange and colleagues did not find that colchicine has any substantial effects on the activity of human NK cells, although they show unusual morphology (e.g., bubbly appearance) in response to colchicine [53]. Katz et al. showed somewhat different results as they demonstrated that colchicine actually inhibits the cytotoxic effects of human NK cells [54]. A few other studies showed that colchicine does not have an effect on adhesion or synapsis formation of human NK cells [55]. Davis and his group determined that NK cell synapse formation is not compromised in the presence of colchicine in a human cell model [56]. The degranulation of human NK cells was increased four-fold in response to a low dose (0.1 μM) colchicine when compared to a high dose (10 μM of colchicine) [57]. This increase in degranulation of NK cells leads to the release of cytolytic granules, an important prerequisite for the NK cell-mediated cytotoxicity [57].

The effects of vinblastine on NK cells are somewhat controversial. Carpén and colleagues reported that, unlike colchicine and taxanes, vinblastine does not impede human NK cell function [58]. In contrast, the Markasz group found that vinblastine inhibits human NK cell-mediated tumor-cell killing, without causing changes in cellular morphology [46]. Further, these authors found that other members of the vinca alkaloid group including vincristine and vinorelbine also inhibit NK cell-mediated tumor-cell killing, albeit less effective than vinblastine [46]. Studying with a pre-clinical mouse model, the Kang group found that vinblastine can enhance or suppress the NK cell activity, depending on the doses used: 1–10 μg (per kg of body weight) enhanced NK cytotoxic activity, while 0.1 μg suppressed it [59]. Clearly, further research is required to sort out this controversy and confusion associated with the vinca alkaloid group on NK cell activities.

10. Regulation of Dendritic Cells

Dendritic cells are the only APC with the ability to stimulate naïve T cells, through which they can activate primary immune responses [60]. Dendritic cells are differentiated from a diverse

lineage, and are found in small populations dispersed in various locations in the body, most commonly in the lymphatic regions [60]. Dendritic cells have an efficient mechanism to stimulate naïve T cells [60]. To activate T cells, dendritic cells require co-stimulators such as CD40, CD80, and CD86 [60]. While naïve T cells are the primary cell population modulated by dendritic cells, memory T cells are also effectively stimulated by them [60]. In addition, dendritic cells are important cytokine producers, ultimately helping to elicit immune responses.

Comparative phenotypic studies have revealed that dendritic cells are derived predominantly from myeloid or plasmacytoid cells [61]. Myeloid dendritic cells express CD11b, CD11c, CD13, and CD33 cell surface antigens, while plasmacytoid dendritic cells express CD123, CD303, and CD304 antigens [61].

Dendritic cells are present in the tumor microenvironment (TME), where they can interact with antigens present on dead tumor cells or nibble at living tumor cells, leading to the activation of T cells [62]. However, tumor cells have the ability of preventing antigen presentation through various mechanisms. First, tumors can alter the typical differentiation process of monocytes by differentiating them to macrophages, rather than to dendritic cells, thus preventing the activation of tumor-specific T cells [62]. Tumors can also impede dendritic cell maturation through the secretion of IL-10 [62]. A recent study demonstrated that the infiltration of CD103+CD11b dendritic cells inhibit IL-12 expression through IL-10 secretion in a breast cancer model [63]. As a result, antigen-specific T cell responses may be hindered. Tumor derived factors can also impede dendritic cell maturation [62]. Aspord et al. showed that TSLP, a cytokine and tumor derived factor, induces the expression of OX40 ligand in dendritic cells, resulting in the T-helper cells accelerating tumor development through the release of IL-4 and IL-13 [64]. These interleukins play a critical role in abrogating tumor cell apoptosis and promoting tumor cell proliferation [64]. One of the major mechanisms by which dendritic cells are impaired during anti-tumor immunity is the result of lipid accumulation in dendritic cells [63]. Dendritic cells with elevated lipid content are not able to effectively stimulate T cells or display tumor associated antigens [65].

11. Responses of Dendritic Cells to Microtubule Inhibitors

While paclitaxel is often used to treat breast, ovarian, and prostate cancers, its high doses have been shown to be immunosuppressive. Ferrari and colleagues demonstrated that cytotoxic levels of paclitaxel on dendritic cells can lead to a decrease in dendritic cell mobilization [65]. However,

low doses of paclitaxel (200 mg per m^2 body) seem to enhance the immunostimulatory activity of dendritic cells [66]. Paclitaxel also interacts with TLR-4 (an LPS receptor) in a murine model, thus positively effecting cell maturation and promoting an immune response [67]. John et al. demonstrated that there is a dose-dependent relationship between dendritic cell proliferation and the subsequent T-cell activation when treated with paclitaxel [68]. They found that cytokine gene expression was down-regulated and chemokine secretion was lacking in dendritic cells treated with paclitaxel, the latter may suggest a decrease in dendritic cell migration in the lymphatic system [69].

Wen et al. demonstrated the importance of functional microtubules for antigen processing by dendritic cells [69]. For example, colchicine was found to promote dendritic cell maturation and antigen-cross presentation [69]. Consistent, the treatment of B16F10 mouse melanoma cells with 2.5 μM colchicine elicited responses of CD4+ and CD8+ T cells [69]. The treatment also induced the expression of damage-associated molecular patterns and tumor-associated antigens [69]. Mizumuto, et al. demonstrated that the expression of MHC II markers was markedly increased following treatment with colchicine in the CD34+ murine progenitor cells [70]. The CD40, CD80 and CD86 co-stimulatory molecules are also up-regulated in response to colchicine [70]. Furthermore, this group also found that dendritic cells treated with colchicine show elevated levels of IL-6, IL-8 and macrophage inflammatory proteins (namely 1α and 3α), without showing the expression of TNF-α [70]. The study also demonstrated an increase in the uptake of FITC-DX, indicating an increase in endocytotic activity in dendritic cells treated with colchicine [70].

Similarly to colchicine, vinblastine induces dendritic cell maturation [71] (Figure 2). Furthermore, vinblastine also inhibits suppressor T cells in tumor microenvironment of a mouse model [71]. Tanaka et al. found that the treatment of mouse bone marrow (BM) derived dendritic cells with vinblastine (ranging from 0.1 to 1.0 μM) leads to increases in the levels of IL-12, IL-6, and IL1-β cytokines as well as CD40, CD80, and CD86 co-stimulatory molecules [72]. In addition, the same treatment of dendritic cells also leads to an increase in the level of MHC II, and consequently, an increase in T-cell stimulatory activity [72]. The authors further alluded to the mechanism by which vinblastine could potentiate dendritic cell maturation, leading them to conclude that there are direct and indirect mechanisms involved in the maturation process in response to vinblastine. The latter may occur through a secondary activation of BM-DC in vinblastine pre-treated cells [72]. Similarly to colchicine treatment, the administration of

vinblastine augments the endocytotic capability of dendritic cells, but to a greater extent than colchicine [72]. Most of the pre-clinical studies investigating the effects of tubulin-targeting agents demonstrated that they can induce dendritic cells activity, as illustrated in Figure 2.

12. The Art of Combining Tubulin Inhibitors and Immunotherapy

Historically, much effort has been undertaken into understanding the roles and impacts of various tubulin-targeting agents in the context of cancer treatments. However, targeting tubulin alone shows only limited success. Many studies have shown the potential benefits of combination of anti-microtubule agents with other therapeutics, especially with immunotherapy [73,74]**.** Immunological therapeutics such as checkpoint blockades show tremendous potential for the control of tumors. However, immunotherapy alone is thus far more effective for only relatively small subpopulations of patients for certain types of cancer [73]. Under normal physiological conditions, immune checkpoints prevent autoimmunity by inhibiting dendritic cell activation of T cells [74,75]. Immune checkpoints may also act by causing T cell exhaustion at sites of inflammation [74,75]. In a way, tumor cells cleverly use this physiological phenomenon to protect them from immune surveillance mechanisms.

Essentially, all of the currently available checkpoint blockades are monoclonal antibodies that can expose tumor cells to the T cell-based immune surveillance mechanism [75]. By enhancing or eliciting immune responses that are often down-regulated in cancerous cells, tumor cells can be selectively eliminated. However, for one reason or another, this seemingly straight forward mechanism does not always work. A promising new approach may be a chemo-immuno combinational approach. There are currently >200 clinical trials involving immune checkpoint inhibitors in conjunction with chemotherapy [74].

Pembrolizumab and nivolumab are common checkpoint inhibitors used to treat non-small cell lung cancer (NSCLC). Numerous clinical trials demonstrate the combination of tubulin inhibitors with the previously stated checkpoint inhibitors for the treatment of non-small-cell lung cancer [76–78]. One clinical study completed by Gadgeel and colleagues, concluded that the combination of pembrolizumab with paclitaxel and carboplatin is feasible and yielded clinically significant positive results regardless of pembrolizumab dose or PD-L1 status in NSCLC patients [78]. This is one of many clinical trial studies that demonstrates the importance and clinical impact of combining tubulin inhibitors with immunotherapies for the treatment of cancer.

13. Concluding Remarks

As reviewed above, the combination of tubulin-targeting anticancer agents and immune therapy appears to be especially promising, as several lines of evidence suggest that agents functioning as anti-polymerization and anti-depolymerization of microtubules can enhance the body's immune response. The next logical step would be a better understanding of the molecular mechanism about how different tubulin-targeting agents can enhance different immunotherapeutic agents in various tumor environments. Undoubtedly, this knowledge will eventually bring about highly effective cancer therapies in individual patients.

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