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Exploring the therapeutic potential of natural products in the treatment of epidermolysis bullosa acquisita

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Zusammenfassung

Hintergrund: Epidermolysis bullosa acquisita (EBA) ist eine Autoimmunerkrankung, die durch subepitheliale Blasenbildung der Haut und der Schleimhäute gekennzeichnet ist. Frühere Studien haben gezeigt, dass sowohl die nicht-entzündliche als auch die entzündliche Form der EBA durch pathogene Autoantikörper gegen Typ-VII-Kollagen (COL7) sind, die eine vermittelt wird. Kürzlich konnte von unserer Arbeitsgruppe nachgewiesen werden, dass die durch den COL7-anti-COL7-IgG-Immunkomplex (IC) induzierte Adhäsion von Neutrophilen ein Schlüsselschritt für die Gewebeschädigung und die dermal-epidermale Trennung ist und damit ein neues therapeutisches Ziel für EBA darstellen könnte. Ziel dieser Studie war die Identifikation von chemischen Verbindungen, die eine IC-induzierte Neutrophilenadhäsion zu blockieren und damit den durch anti-COL7-IgG vermittelten Gewebeschaden zu hemmen.

Methoden: In einem ersten Schritt wurden 800 Substanzen einer Naturstoff-Bibliothek auf ihre Kapazität zur Hemmung der Zelladhäsion von Neutrophilen *in vitro* in einem Echtzeit-Messsystem hin untersucht. Positiv evaluierte Kandidaten wurden nachfolgend darauf geprüft, ob sie ihre Wirkung in einem eindeutigen Dosis-Wirkungsverhältnis vermitteln und ob sie auf Neutrophile einen zytotoxischen Effekt haben. In einem weiteren Schritt wurde die funktionelle Spezifität der positiv evaluierten Kandidaten für die Blockade der Adhäsion von Neutrophilen untersucht. Final wurde die therapeutische Wirksamkeit der Kandidatenverbindungen mit Hilfe eines Ex-vivo-Modells der EBA bestimmt.

Ergebnisse: Im Primärscreening wurden 11 Verbindungen aus den 800 getesteten Naturstoffen identifiziert, bei denen sich eine Hemmung der IC-induzierten Neutrophilenadhäsion nachweisen ließ. Unter den 11 Verbindungen wiesen 5 Produkte, nämlich Luteolin peracetate, Plumbagin, Digitonin, Gossypol und Gossypolon, in Konzentrationskinetiken eine signifikate Dosis-Wirkungs-Beziehung auf. Von den 5 Kandidaten wiesen 3 Substanzen, nämlich Luteolin peracetate, Gossypol und Gossypolon, keine toxischen Nebenwirkungen auf Neutrophile auf. Darüber hinaus inhibierten sowohl Luteolin als auch Gossypolon signifikant die anti-COL7 IgG-vermittelte dermal-epidermale Trennung im ex vivo-Modell für EBA.

Schlussfolgerung: Durch das Screening einer Naturstoffbibliothek mit 800 Naturstoffen konnten in dieser Studie Luteolin peracetate und Gossypolon als neue potenzielle Therapeutika zur Behandlung der EBA identifiziert werden, deren weitergehende Untersuchung in Studien *in vivo* erfolgversprechend erscheint.

Summary

Background: Epidermolysis bullosa acquisita (EBA) is an autoimmune disease characterized by sub-epithelial blistering of the skin and mucosal membranes. Previous studies have demonstrated that anti-type VII collagen (COL7） IgG represent pathogenic autoantibodies which mediate both, the non-inflammatory or inflammatory form of EBA. Recently, we reported that neutrophils adhesion induced by COL7-anti-COL7 IgG immune complex (IC) is a key step for tissue damage and dermal-epidermal separation in experimental EBA, providing a novel therapeutic target for the disease. In this study, I aimed to identify chemical compounds that are capable of inhibiting IC-induced neutrophil adhesion and anti-COL7 IgG mediated tissue damage.

Methods: First of all, 800 compounds derived from a natural product library were evaluated for their capacity to inhibit cell adhesion of neutrophils *in vitro* in a real-time measurement system.. Second, natural products identified in first step were evaluated in dose-response assessment, and only candidates that showed significant dose-response relationships were selected for cytotoxic effects on neutrophils. In a further step, the functional specificity of the positively evaluated candidates for blocking neutrophil adhesion was investigated. Finally, the therapeutic efficacy of candidate compounds was determined using an *ex vivo* model of EBA.

Results: The primary screen identified 11 compounds from the 800 natural products tested that demonstrated inhibition of IC-induced neutrophil adhesion. Among the 11 compounds, 5 products, namely luteolin peracetate, plumbagin, digitonin, gossypol, and gossypolone, exhibited a significant dose-response relationship in concentration kinetics. Of the 5 candidates, 3 compounds, namely luteolin peracetate, gossypol, and gossypolone, did not exhibit toxic side effects on neutrophils. Furthermore, both luteolin peracetate and gossypolone significantly inhibited the anti-COL7 IgG mediated dermal-epidermal separation in an *ex vivo* model for EBA.

Conclusion: By screening a natural product library containing 800 natural compounds, this study identified luteolin peracetate and gossypolone as inhibitors of neutrophil functions in vitro and ex vivo. They represent potential new therapeutics for the treatment of EBA and will be investigated further in studies *in vivo*

1 Introduction

1.1 Epidermolysis Bullosa Acquisita (EBA)

1.1.1 Diagnosis of EBA

Epidermolysis bullosa acquisita (EBA) was first mentioned as a descriptive clinical diagnosis at the end of the 19th century by G.T. Elliot. EBA presents a type of epidermolysis bullosa which occurs in adulthood without showing a hereditary origin for its occurrence (Elliot, George T. 1895).

The first diagnostic criteria for EBA was proposed by Roenigk et al. at 1971, which contains the following 4 items, (1) onset of the clinical symptoms in adulthood, (2) no hereditary evidence for the existence of epidermolysis bullosa dystrophica, (3) exclusion of other bullous diseases, and (4) sharing the similar clinical lesions of epidermolysis bullosa dystrophica (Roenigk, Ryan, and Bergfeld 1971). In 1988, David Woodley and his colleagues identified that anti-type VII collagen (COL7) IgG as the pathogenic autoantibody for EBA (D. T. Woodley et al. 1988). This breakthrough and subsequent research advances in the field significantly contributed to the improvement of the diagnostic criteria for the disease. According to the International Bullous Diseases Group (Yamagami 2018)**,** criteria for EBA diagnosis include combinations of the following tests: (1) a bullous disorder within the defined clinical spectrum; (2) histopathology revealing a subepidermal or subepithelial blister; (3) a positive direct immunofluorescence (DIF) microscopy of perilesional skin or MM with linear IgG, complement component 3 (C3), IgA and/or IgM deposits within the epithelial basement membrane zone (BMZ); (4) detection of circulating autoantibodies against COL7 by immunoblotting (IB), enzyme-linked immunosorbent assay (ELISA) and/or indirect immunofluorescence (IIF) microscopy on COL7-expressing human cells; (5) labelling anchoring fibrils (AFs) by indirect Immunoelectron microscopy (IEM) or negative IIF microscopy on COL7-deficient skin; (6) a ‘u-serration’ pattern by DIF microscopy; (7) direct IEM of perilesional skin demonstrating immune deposits within AFs zone ± the lower lamina densa (LD); (8) *in vivo* bound immune deposits below type IV collagen by fluorescent overlay antigen mapping (FOAM); (9) alternatively to items (4)–(8), dermal labelling by DIF and/or IIF on salt-split skin (SSS). Ideally, when a patient shows putative EBA disease (criterium 1) to exhibit a subepidermal bulla by histology (criterium 2 optional), positive DIF microscopy (criterium 3) and an ELISA (or another serological test) showing that the patient's serum autoantibodies against COL7 (criterium (4) or (5)), a highly probable diagnosis of EBA can be made and no further tests need to be done for confirmation. In case that a patient with EBA lacks circulating autoantibodies and therefore IIF, SSS IIF and ELISA are negative, then the diagnosis of EBA could be considered definitive if criteria (1) and (3) and at least one of criteria (6)–(8) are present [criterium (2) is optional. Finally, if tests (6)–(8) cannot be done, a diagnosis of EBA is possible if items criteria (1), (3) and (9) are present, but the diagnosis has to be confirmed by exclusion of autoimmunity against laminin 332 or the p200/laminin γ1 chain (Yamagami 2018). According to the presence of inflammatory symptoms, generally EBA can be classified into two major clinical forms: non-inflammatory and inflammatory EBA (Ishii, Hamada, et

al. 2010; Gupta, Woodley, and Chen 2012; Schmidt and Zillikens 2013). The noninflammatory EBA, also known as the classical mechanobullous variant, was firstly depicted by Roenigk in the 1970s (Roenigk, Ryan, and Bergfeld 1971; J. H. Kim, Kim, and Kim 2011). According to Iwata et al., approximately one-third of EBA patients are diagnosed as classical mechanobullous variants (Iwata et al. 2018). Clinically, non-inflammatory EBA is characterized by skin fragility, tense blisters on non-inflamed site, scarring and milia formation specialized on trauma-prone areas (Roenigk, Ryan, and Bergfeld 1971; J. H. Kim, Kim, and Kim 2011). Nearly two-thirds of EBA patients suffer from inflammatory variants of the disease (Iwata et al. 2018). According to the similarity to clinical manifestations of other autoimmune bullous dermatoses, inflammatory EBA can be further categorized into two subtypes, bullous pemphigoid (BP)-like and mucous membrane pemphigoid (MMP)-like EBA (W. Ray Gammon et al. 1984; Kurzhals 1991; Zambruno et al. 1994; Dahl 1979).

1.1.2 Epidemiology of EBA

EBA is a rare disorder, with a much lower incidence and prevalence than other autoimmune bullous diseases (Ishii, Hamada, et al. 2010; Gupta, Woodley, and Chen 2012; Schmidt and Zillikens 2013). The incidence of EBA was first estimated by a prospective study in 1995 (Bernard et al. 1995), where Bernard and colleagues reported that the annual incidence of EBA range from 0.17 to 0.26 per million people per year among three French regions. In 2002, by analysing the clinical data from National Skin Center of Singapore, Wong et al. reported an estimated incidence of EBA as 0.5 per million people per year, which is approximately twice as high as the incidence in western Europe (Wong and Chua 2002). In addition to the disease incidence, the prevalence of EBA has also been investigated. By analyzing the database of a major German health insurance company, Hübner et al. reported that the prevalence of EBA in Germany is 2.84/million inhabitants (Hübner et al. 2016).

EBA is almost exclusively diagnosed in adults, only with rare exceptions in children (Trigo-Guzmán et al. 2003; Arpey et al. 1991). The onset age of EBA varies among populations. For example, the median age of onset for EBA in Korean population is 44.0 years (J. H. Kim, Kim, and Kim 2011), while those values in Singapore and Germany are 68 years (Wong and Chua 2002) and 60 years (Hübner et al. 2016), respectively.

In addition to disease incidence, prevalence and age of onset, susceptibility to EBA also varies among populations. For example, Zumelzu et al. reported that black-skinned patients are more susceptible to EBA than Caucasians (Zumelzu et al. 2011). Regarding the effect of gender on the development of the disease, significant differences in disease incidence has been observed between females and males (Wong and Chua 2002) (Hübner et al. 2016) (J. H. Kim, Kim, and Kim 2011).

With regard to the disease comorbidity, it has been well documented that EBA is associated with other pathological conditions, including inflammatory bowel disease (IBD), systemic lupus erythematosus, chronic lymphocytic leukemia, rheumatoid arthritis, pulmonary fibrosis and diabetes (M. Chen et al. 2012; Hundorfean, Neurath, and Sitaru 2010; M. Chen et al. 2002). For example, Chen and colleagues reported that approximately 25% of patients with EBA also suffer from IBD, and about 70% of patients with Crohn's disease are positive for autoantibodies to COL7 (M. Chen et al. 2012, 2002). The association of EBA with IBD is also supported by experimental evidence where the transfer of anti-COL7 IgG induces both skin and intestinal inflammation in mice (Ishii et al. 2011).

1.1.3 Histopathology and immunopathology of EBA

1.1.3.1 Histopathology of EBA

The dermal-epidermal junction (DEJ) which joins the epidermal and the dermal layers of the skin is the key structure to keep the integrity of the normal skin integrity. Anchoring fibrils, a main component of DEJ, is composed of type IV collagen and type VII collagen and plays a crucial role in tethering the basal lamina to the underlying dermis function of DEJ (Hashimoto et al. 2012; Keene et al. 1987). As a consequence, the dysfunction of type VII collagen could lead to an impaired skin integrity.

In the biopsy obtained from the lesional skin of patients with EBA, the histological features vary according to the different phases of the disease. In early phases, vacuolar alterations are often observed along the DEJ with subjacent papillary dermal edema (M. Chen et al. 2012). In later phases, the separation between dermal and epidermal layers can be found easily in most cases. In the inflammatory form of EBA, clinical manifestations are associated with dermal inflammatory infiltrate which is predominantly composed of neutrophils, while such dermal infiltration is not shown in classical mechanobullous EBA (Lever, n.d.). In addition, fibrosis and milia can be observed in classical mechanobullous clinical variant cases (Bernard Ackerman, n.d.). Finally, the absence of anchoring fibrils, an electron-dense band directly underneath the lamina densa, can be detected in skin biopsy of patients with EBA with electron microscopy (Richter and McNutt 1979; Ray et al. 1982).

1.1.3.2 Immunopathology of EBA

The first milestone of the immunopathology of EBA was achieved around 40 years ago. In their study, Yaoita and colleagues reported linear depositions of antibodies and complement along the dermal-epidermal junction in the biopsy obtained from EBA patients (Yaoita et al. 1981). Subsequently, Woodley and his team identified COL7 as the autoantigen of EBA (D. T. Woodley et al. 1988, 1984).

COL7 is composed of three identical alpha chains each of which consists of a large 145 kDa amino-terminal non-collagenous domain (NC1), and a small 34-kDa carboxyl-terminal non-collagenous domain (NC2) (Keene et al. 1987). COL7 is a major component that composing of anchoring fibrils in the DEJ. Because various sub-modules are located within the 145 kDa amino-terminal, the NC1 domain plays a critical role in the binding of COL7 to other BMZ components and stabilizing the adhesion of the BMZ to the underlying dermis (M. Chen et al. 2012). Furthermore, the NC1 domain also contains antigenic epitopes of anti-COL7 IgG, which has been demonstrated by several studies (Lapiere et al. 1993; W. R. Gammon et al. 1993) (M. Chen et al. 2007).

With various experimental models, it has been convincingly shown that anti-COL7 IgG are pathogenic autoantibodies in EBA (C. Sitaru et al. 2006; C. Sitaru 2007; David T. Woodley, Remington, and Chen 2007). Although it remains largely unclear how autoantibodies against COL7 are generated, an association of HLA-DR2 with EBA suggests that those pathogenic autoantibodies are generated in a T-cell dependent manner (W. Ray Gammon et al. 1988). This association of EBA with MHC II molecules is further supported by evidence that the development of the mouse model for EBA is dependent on H2s haplotype (Ludwig et al. 2011). Furthermore, a critical role of T cells in the development of EBA has been demonstrated by several studies. For instance, Kasperkiewicz M, et al. for the first time reported that COL7-specific T cells are presented in peripheral blood of EBA patients (Kasperkiewicz et al. 2011). Moreover, Sitaru and colleagues reported that T cell-deficient mice are resistant to experimental EBA (A. G. Sitaru et al. 2010), suggesting an indispensable role of T cells in the disease pathogenesis. Regarding the contribution of T cell subtypes, it has been demonstrated that CD4+ T cells, but not CD8+ T cells, are the key player in the development of the disease. (A. G. Sitaru et al. 2010).

Once anti-COL7 IgG are generated and released, they circulate in peripheral blood. During circulation, the neonatal Fc receptor (FcRn) plays a critical role in prolonging the half-life of anti-COL7 IgG (T. T. Kuo et al. 2010). Once the circulating anti-COL7 IgG migrate into the skin, the binding of those autoantibodies to the autoantigen at the DEJ is able to mediate disease manifestations. Upon the binding to COL7 at the DEJ, autoantibodies against COL7 are able to induce symptoms in at least two ways. In the inflammatory EBA, the binding of autoantibodies to COL7 forms IC which mediates subsequent inflammatory cell infiltration in a complement dependent manner (C. Sitaru et al. 2006; Hammers et al. 2011). Thereafter, infiltrated neutrophils migrate to DEJ and are activated by IC via the Fcγ receptors (C. Sitaru, Kromminga, et al. 2002c) (Kasperkiewicz et al. 2012). Finally, the activated neutrophils act as the executor and cause tissue damage as well as dermal-epidermal separation (Shimanovich et al. 2004). (Chiriac et al. 2007; Mihai et al. 2007; C. Sitaru et al. 2005). In the non-inflammatory EBA, the binding of autoantibodies to COL7 does not initiate inflammatory responses, and the development of skin symptoms seems dependent on mechanical trauma (Roenigk, Ryan, and Bergfeld 1971; J. H. Kim, Kim, and Kim 2011).

1.1.4 Current Treatments of EBA

Because of low disease prevalence, no controlled clinical trials on the treatment has been performed for EBA. Therefore, current options for EBA treatment are solely based on the clinical expertise by clinicians specialized in AIBD (Ludwig 2013).

Similar to other AIBDs, systemic corticosteroids are widely used for the treatment of EBA (Iwata et al. 2018). Treatment with corticosteroids leads to significant improvement in symptoms such as skin fragility, vesicles, bullae, or erosions and scarring with milia (Witte et al. 2016). Due to the numerous and severe side effects of high dose systemic corticosteroids as monotherapy, low dose of corticosteroids combined with steroid-sparing agents, including colchicine, diaminodiphenyl sulfone (DDS, dapsone), methotrexate (MTX), azathioprine (AZA), cyclosporine (CSA), and cyclophosphamide (CPA) have been applied in the treatment of EBA (Koga et al. 2018). Monotherapy with steroid-sparing agents has also been applied for the treatment of EBA. For example, colchicine is recommended by some experts as the first-line treatment of EBA due to its fewer side effects compared with other medications (Ludwig 2013). In addition, cyclosporine and dapsone have been applied in EBA patients as a monotherapy (Khatri, Benghazeil, and Shafi 2001; Maize and Cohen 2005; Hughes and Callen 2001), and approximately half of EBA cases treated with cyclosporine or dapsone showed remission (J. H. Kim, Kim, and Kim 2011). However, the long term effects of these monotherapies are limited due to their severe side effects and the high recurrence rate after the withdrawal of the medication (Iwata et al. 2018).

Besides the above mentioned small molecule chemicals, some biological drugs have been applied for the treatment of EBA over the last two decades (Koga et al. 2018). One example is the application of high-dose intravenous immunoglobulin (IVIG) (Ahmed and Gürcan 2012). Originally, IVIG has been used as a substitution treatment for patients diagnosed with antibody deficiencies and severe infections (van der Burg and Gennery 2011). Later, high-dose IVIG has been applied as an effective therapy for some autoimmune disorders, such as immune thrombocytopenia (ITP) and pemphigus disease (Amagai et al. 2009). In 2010, a satisfactory clinical response was reported in patients with EBA, supporting that IVIG is an effective treatment option for the disease (Ishii, Hashimoto, et al. 2010). Of note, the effectiveness of high-dose IVIG varies among patients with clinical phenotypes of EBA (Iwata et al. 2018). Another biological drug for EBA is Rituximab, an anti-human CD20 monoclonal antibody, which has been shown to be effective in the treatment of many autoimmune disorders (MacIsaac et al. 2018). In addition, removal of autoantibodies using plasmapheresis and immunoadsorption has been applied to treat patients with EBA (Ludwig 2013).

Taken together, many drugs have been applied for the treatment of EBA, but the current treatment of the disease remains challenging. Therefore, further understanding of the pathogenesis of the disease is required for aiding us to identify novel potential therapeutics.

1.1.5 Experimental models of EBA

Experimental models of human diseases are invaluable tools in modern biomedical research. They not only help us to understand the disease pathogenesis, but also aid us to search for novel potential therapeutics for human diseases (Iwata et al. 2015; Yu 2015). So far, three elaborate experimental systems of EBA, including in vivo models, an ex vivo model as well as an in vitro experimental system have been established for resembling EBA. These experimental models have greatly improved our understanding of the pathogenesis of EBA and thus are useful for the discovery of novel potential therapeutic targets.

1.1.6 In vivo models of EBA

Based on strategies used to induce disease, mouse models of autoimmune diseases can be categorized into two groups, active and passive models (Yu and Petersen 2018). In the active model, mice are immunized with antigens and consequently show autoimmune responses and disease manifestations. By contrast, mice in the passive models are transferred with autoimmune components such as autoantibodies or autoreactive lymphocytes and thus consequently develop disease symptoms. So far, both passive and active mouse models have been established for EBA (C. Sitaru 2007).

As a disorder caused by anti-COL7 IgG, mouse models of EBA can be induced by the transfer of either anti-murine COL7 IgG or anti-human COL7 IgG. The first passive model for EBA was reported in 2005 by a German research group (C. Sitaru et al. 2005). To induce experimental EBA, Sitaru and his colleagues firstly generated rabbit anti-murine COL7 IgG by immunizing rabbits with recombinant fragments of the NC1 domain of murine COL7, and then transferred the rabbit anti-murine COL7 IgG into mice. After the transfer, both Balb/c and C57BL/6 immune competent mice developed inflammatory EBA-like skin symptoms including inflammation, erosions, and dermo epidermal separation (C. Sitaru et al. 2005). Passive mouse models induced by transfer of anti-human COL7 IgG were also reported in 2005 by Woodly and colleagues (David T. Woodley et al. 2005). In their study, rabbit anti-human COL7 IgG were generated by immunizing rabbits with the whole NC1 domain of human type VII collagen and then transferred into SKH1 mice. The clinical manifestations in SKH1 mice were observed at day 4 after the first IgG injection and lasted more than one month. The successful establishment of the passive mouse model induced by rabbit anti-human COL7 IgG suggests a cross-reactivity between anti-human COL7 IgG and murine COL7. This notion is further supported by another

passive model which is induced by transferring IgG fractions isolated from patients with EBA into SKH1 mice (David T. Woodley et al. 2006). The recipient SKH-1 mice developed clinical, histological, immunological, and ultrastructural characteristics of the disease (David T. Woodley et al. 2006).

Although passive mouse models for EBA provide the possibility to study the pathomechanism of how anti-COL7 IgG induces tissue inflammation and damage, they are not helpful for our understanding of the afferent phase of the disease. Thus, an active model induced by immunization with COL7 is required. In 2006, Citaru et al. reported a mouse model for EBA induced by immunizing mice with a recombinant fragment of NC1 domain of murine COL7 (C. Sitaru et al. 2006). In this active model, the development of experimental EBA is strain-dependent. Among various strains, only SJL/J is susceptible to the development of the disease, while C57Bl/6, Balb/c and SKH1 are resistant, suggesting the disease development is dependent on the MHC II haplotype of H2s. This notion was validated in a later study where Ludwig and colleagues showed that C57BL/10.s (H2s) mice are susceptible to the induction of experimental EBA (Ludwig et al. 2011). This strain-dependent susceptibility is supported by another study published in the same year, where Chen et al. reported that immunization with recombinant NC1 domain of murine COL7 induced the production of anti-murine COL7 IgG but failed to induce clinical symptoms (L. Chen et al. 2006). Furthermore, in contrast to wild type C57BL/6 mice, FcγRIIB deficient C57BL/6 mice are susceptible to the disease induction, demonstrating a protective role of FcγRIIB (C. Sitaru et al. 2006).

1.1.7 Ex vivo Models of EBA

A hallmark of EBA is dermal-epidermal separation, a consequence of damage mediated by neutrophils when they are activated by the COL7-anti-COL7 IgG immune complex (IC). This neutrophil mediated dermal-epidermal separation can be resembled with skin cryosections, anti-COL7 IgG and neutrophils, providing an *ex vivo* model for EBA (C. Sitaru, Kromminga, et al. 2002b; Herrero-González et al. 2006; Shimanovich et al. 2004). The first *ex vivo* model for autoimmune disorders was established by Yamamoto and his colleagues to study the interaction between IC and neutrophils in glomerulonephritis (Yamamoto et al. 1979). Later the concept was applied to generate *ex vivo* models for various autoimmune bullous diseases, such as bullous pemphigoid and EBA (W. Ray Gammon et al. 1981; W. R. Gammon, Inman, and Wheeler 1984). In an *ex vivo* model of EBA, cryosections of human or murine skin biopsies are incubated with corresponding anti-human or murine COL7 IgG, and further incubated with neutrophils to induce dermal-epidermal separation. The cryosection-based *ex vivo* model not only present an excellent tool for investigating the mechanism behind the neutrophil mediated tissue damage, but also provides a platform for screening potential therapeutic compounds (Chiriac et al. 2007; Kulkarni et al. 2011; Yu et al. 2014; Kemmer et al. 2015).

1.1.8 In vitro system of neutrophil activation

In experimental models of EBA, activation of neutrophils by COL7-anti-COL7 IgG IC is a key step for consequent tissue damage and dermal-epidermal separation (Chiriac et al. 2007; Yu et al. 2018). Upon activation by IC, neutrophils adhere to the target tissue, release proteinase and generate reactive oxygen species (ROS), which are essential biological processes in tissue damage in EBA (Chiriac et al. 2007; Yu et al. 2018). The IC-mediated neutrophil activation in experimental EBA can be determined in vitro. In this in vitro neutrophil activation system, surface coated with COL7 are incubated with anti-COL7 IgG to form ICs, then neutrophils are subsequently added to the IC. The IC-mediated neutrophil activation can be quantified by detecting adhesion, ROS production and release of proteinases (Yu et al. 2010). This in vitro system provides a high throughput platform for the investigation of IC-mediated neutrophil activation (Kulkarni et al. 2011; Recke et al. 2010; Yu et al. 2014).

Taken together, in vivo and ex vivo models of EBA as well as the in vitro neutrophil activation system provide complementary research methods for exploring the pathogenesis of EBA.

1.2 Neutrophils in EBA

As the most abundant circulating leukocytes in human peripheral blood, neutrophils not only play a primary role in the first line of protection against pathogenic microorganisms, such as bacterial and fungal, but also are essentially involved in the development of several autoimmune diseases, for example, rheumatoid arthritis, systemic lupus erythematosus, autoimmune vasculitis and autoimmune bullous diseases (Németh and Mócsai 2012). Notably, accumulating evidence suggests that neutrophils also play an essential role in the development of EBA and its experimental models (Ludwig 2013). As a complicated process, the neutrophil-mediated tissue damage consists of several essential steps, including recruitment of neutrophils into the skin, binding of neutrophils to IC, activation of neutrophils and tissue damage (C. Sitaru et al. 2006; Hammers et al. 2011; C. Sitaru, Kromminga, et al. 2002c; Shimanovich et al. 2004).

1.2.1 Neutrophils as the executor of tissue damage

The binding of anti-COL7 IgG to COL7 in DEJ forms IC, which is able to activate the complement system and thus produce C5a a strong chemoattractant (Koga et al. 2018). In antibody transfer-induced EBA, it has been shown that C5a is indispensable for the infiltration of neutrophil and blister formation (Mihai et al. 2007; Karsten et al. 2012), suggesting an essential role of C5a in the recruitment of neutrophils. Beside C5a, lipid mediator leukotriene B4 (LTB4) has been identified as another important chemotactic factor for neutrophil recruitment in antibody transfer-induced EBA (Sezin et al. 2017).

During the migration from circulation to the skin, the extravasation of neutrophils is mediated by LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) of neutrophils and ICAM-1 of endothelial cells (Hyun et al. 2019).

Once recruited into the skin, neutrophils bind to the COL7-anti-COL7 IgG IC via Fc gamma receptors (FcγR). Several studies have demonstrated that the tissue damage and blister formation in experimental EBA are dependent on FcγRs on neutrophils (Yuasa et al. 1999; Blank et al. 2005; Nimmerjahn and Ravetch 2008), suggesting an essential role of neutrophil activation mediated by IC. In 2012, Kasperkiewicz and colleagues conducted a comprehensive study to determine the role of different FcγRs in an antibody transfer-induced model of EBA (Kasperkiewicz et al. 2012). Their results showed that there is hyperexpression of FcγRIV mRNA and the hypo expression of FcγRIIB mRNA in infiltrated neutrophils in the lesional skin of mice injected with anti-murine COL7 IgG. Furthermore, using knockout mice of blocking monoclonal antibodies, Kasperkiewicz and colleagues could show that i) typical presentation of experimental EBA mice was observed after the injection of anti-COL7 IgG in mice deficient in FcγRI , FcγRIII or both FcγRI and FcγRIII , ii), mice deficient in FcγRIV or treated with FcγRIV function-blocking antibody were effectively protected from EBA. Based on these findings, Kasperkiewicz et al concluded that FcγRIV on neutrophils is the key receptor for the IC-mediated neutrophil activation and subsequent tissue damage. In line with this finding, human FcγRIIIA, the closest human relative to mouse FcγRIV, has also been suggested to play a role in an ex vivo model of BP (Yu et al. 2010).

As the first line of innate immune cells, neutrophils are well equipped with reactive oxygen species (ROS)-producing enzymes and proteases aiming to degrade microbial proteins or cell wall components (Kaplan 2013). Upon the activation by IC, neutrophils generate a large amount of ROS and release many proteases including MMP9 and elastase. In 2007, Ciriac et al. reported that neutrophil cytosolic factor 1 (Ncf1) deficient mice in which the NADPH oxidase enzyme is dysfunctional were protected from tissue damage after the injection of anti-COL7 IgG in a passive experimental EBA model (Chiriac et al. 2007), suggesting an essential role of neutrophil ROS in the tissue damage in experimental EBA. Furthermore, it has been shown that neutrophil-derived elastase is also required for dermal-epidermal separation induced by autoantibodies from patients with EBA(Shimanovich et al. 2004). Therefore, both ROS and neutrophil elastase are key molecules executing the disease damage in experimental EBA.

1.2.2 Neutrophil adhesion as a novel indispensable process in pathogenesis of EBA

Besides generating ROS and releasing proteinases, adhering to the IC-coupled surface is another hallmark of neutrophil activation (Yu et al. 2018). Given the indispensable role of ROS and elastase in

the pathogenesis of EBA, it is conceivable that the IC-induced neutrophil adhesion also plays a role in the development of the disease.

The first hint for a role of neutrophil adhesion comes from a study published in 2007, where Chiriac and colleagues showed that CD18-/- mice were resistant to the development of antibody-transfer induced EBA, demonstrating that β2 integrins play an essential role in the pathogenesis of the disease (Chiriac et al. 2007). β2 integrins consist of two subunits of heterodimeric transmembrane glycoproteins, an integrin β chain (CD18), and an α chain. According to the unique α chain, β2 integrins are classified into four subtypes, including αLβ2 (CD11a/CD18, LFA-1), αMβ2 (CD11b/CD18, Mac-1), αXβ2 (CD11cCD18), and αDβ2 (CD11d/CD18). The distribution of β2 integrins varies among leukocyte subpopulations. For example, all circulating leukocytes expressing LFA-1, while Mac-1 is mainly detected on neutrophils, monocytes, and macrophages (Futosi, Fodor, and Mócsai 2013; Asada et al. 1991; Pilling et al. 2009). However, as the β2-integrins mediated neutrophil adhesion are involved in various neutrophils related events, As β2 integrin family is involved in several cellular events of neutrophils, including migration from circulation and adhesion to the IC-coupled surface, (Schymeinsky, Mócsai, and Walzog 2007), it needs to be further elucidated whether IC-induced neutrophil adhesion is required for the tissue damage.

To specifically investigate the role of neutrophil adhesion to the IC-coupled surface in the disease, our group utilized the in vitro neutrophil activation system and ex vivo model of EBA (Yu et al. 2018). In vitro, neutrophil adhesion to the surface coated with COL7-anti-COL IgG IC could be not only observed with a microscope but also quantified in a real-time manner using an electronic impedance-based xCELLigence system (Tang et al. 1997; Yu et al. 2010; Scrace et al. 2013). Using the in vitro neutrophil activation modelling system, we have demonstrated that blocking CD18 could specifically inhibit the COL7-anti-COL7 IgG IC-induced neutrophil adhesion, suggesting that the IC-induced neutrophil adhesion is dependent on β2 integrins. This finding is in line with results from previous studies which reported the indispensable role of CD18 in the process of IC-induced neutrophil adhesion (Soriano et al. 1999; Tang et al. 1997; van Spriel et al. 2001). Moreover, we further investigated the role of IC-induced neutrophil adhesion in the tissue damage in an *ex vivo* model of EBA where skin cryosection from mouse ear was incubated with anti-mCOL7 IgG and subsequently with human neutrophils (Yu et al. 2018). Our study demonstrated that anti-CD18 blocking antibodies could prevent the dermal-epidermal separation induced by anti-COL7 IgG (Yu et al. 2018). Given the fact that CD18 blockage does not interfere with IC-induced ROS production and elastase release from neutrophils (Yu et al. 2018), this finding clearly demonstrated that IC-induced neutrophil adhesion plays an indispensable role in tissue damage in the ex vivo model.

Based on a closed-space theory which was suggested more than 30 years ago (E. J. Campbell et al. 1982; Weiss and Regiani 1984), our group proposed a hypothesis explaining how IC-induced neutrophil adhesion contributes to the tissue damage in experimental EBA. According to their hypothesis, a close compartment between neutrophils and the surface is formed by IC-induced neutrophil adhesion, which may protect neutrophil elastase from proteinase inhibitors and thus allows the tissue damage (Yu et al. 2018). Therefore, IC-induced neutrophil adhesion is an indispensable step in the pathogenesis of experimental EBA, and thus it is a promising novel target for the development of new therapies for the disease.

1.3 Natural Products in drug discovery

1.3.1 Values of Natural Product Library

Natural products are defined aschemicals that are biosynthesized with secondary metabolites in various organism, including plants, animals, fungus as well as the creatures from marine environment (Dewick 2002; Maplestone, Stone, and Williams 1992). From the earliest record of natural products for treating diseases in the Ebers Papyrus (2900 B.E.C.) to numerous FDA-approved naturally derived drugs, natural products have been proven to be the most prosperous and significant resource for the new medicines development (Dias, Urban, and Roessner 2012).

Owing to the diversity of secondary metabolites in different organism, natural products show a richer variety of molecular structure than standard combinatorial chemistry. Such widely diverse structures of natural products offer the possibilities for the discovery of lead compounds as medicines(Chin et al. 2006). So far, more than half of FDA-approved drugs are derived from natural products (Kingston 2011). By comparing the natural products recorded in Universal Natural Products Database with FDA approved drugs in 3D digital structure and biology activity, Gu et.al demonstrated the good drug-like properties of natural products and its potential prospect in drug discovery (Gu et al. 2013). It has been reported that certain natural products may play a specific role as ligands of pathogenesis-related cellular targets because of the selectivity of natural products to cellular targets (Lagunin, Filimonov, and Poroikov 2010; Clardy and Walsh 2004). Besides, there are only quit limited bioactivity of natural products are appreciated for the discovery of potential therapy (Cragg and Newman 2013).

1.3.2 Neutrophils as potential target of naturally derived drugs

Neutrophil granulocytes are the most numerous type of peripheral leukocyte in humans bearing an enormous therapeutic potential. Recently, several natural products have been ridentified as potential therapeutics targeting neutrophils. Given an important role of neutrophil in the pathogenesis of

rheumatoid arthritis, effects of plant derived natural compounds on neutrophils have been evaluated ,, and 5 herbal natural products which show beneficial effects such as, antioxidant, anti-inflammatory, antiproliferative and immunomodulatory properties have been suggested as one promising alternative to treat rheumatoid arthritis, (Rosas, Correa, and Henriques 2017) . Among the 5 herbal natural products, methyl gallate shows an inhibitory effects on neutrophil activation and adhesion molecules espression (Rosas et al. 2015; Correa et al. 2016). Also, hydrosols obtained from flowers of Rosa damascena has been demonstrated to be capable to inhibite the LPS-induced neutrophil adhesion(Maruyama et al. 2017). In addition, Wang et al. reported a novel and highly effective neutrophil migration inhibitor by screening approximately one thousand fungal extracts using an in vivo assay (Wang et al. 2014). Taken together, these studies demonstrate the promising prospect of natural products as potential therapeutic compounds targeting neutrophils.

1.4 Hypothesis and Aims

Given that IC-mediated neutrophil adhesion is an indispensable step in the tissue damage of EBA, neutrophil adhesion could be a potential therapeutic target for the disease. Therefore, I hypothesized that the tissue damage in experimental EBA can be attenuated by compounds which are capable of inhibiting IC-mediated neutrophils adhesion. In this study, I aimed to identify chemical compounds that are capable of inhibiting IC-mediated neutrophil adhesion and anti-COL7 IgG-mediated tissue damage.

To reach this aim, I firstly screened a natural product library using the in vitro neutrophil activation system in order to identify potential compounds of interest. The TimTec Natural Products Library composed 800 pure natural compounds from diverse nature material, like plants, bacteria, fungus, and animal sources. According to one report on natural products, the TimTec Natural Product Library is identified one of the most important natural products resources for drug discovery (Füllbeck et al. 2006). After the evaluation of the safety and specificity of the potential candidate, I further evaluated the therapeutic efficacy of the candidates using an *ex vivo* model of EBA. In this study several natural products were identified which are able to inhibit IC-induced neutrophil adhesion and anti-COL7 mediated tissue damage. These substances could be candidates for new therapeutics for the treatment of EBA.

2 Materials and Methods

2.1 Materials

2.1.1 Equipment and consumables

Name	Manufacturer
Absorbance reader	Tecan Trading AG, Switzerland
Microplate Luminometer	EG&G BERTHOLD, Germany
Biological safety cabinet	Thermo Fisher scientific, USA
Centrifuge	Hettich lab technology, Germany
Frozen Centrifuge System	Hettich lab technology, Germany
Vacuum hand controller	VACUUBRAND GmbH & Co. KG, Germany
Pipette controller	VACUUBRAND GmbH & Co. KG, Germany
Vortex mixer	Scientific industries, inc, USA
Water bath	Gesellschaft für Labortechnik GmbH, Germany
Image acquisition system	Nikon Corporation, Japan
Microscope	Carl Zeiss AG, Germany
Cells Incubator	Thermo Fisher scientific, USA
Ultra-low freezer	Thermo Fisher scientific, USA
Scissors and forceps	Karl Hammacher GmbH, Germany
Pipette tips (10µl, 100µl, 1000µl)	Sarstedt, AG&Co, Germany
Frozen section slides	R. Langenbrinck GmbH, Germany
O.C.T. (optimum cutting temperature) compound	Sakura Finetek Europe, Netherlands
Cryosection working station	Thermo Fisher scientific, USA
CASY cell counter	Omni Life Science GmbH & Co. KG, Germany
Laminar flow hood	Thermo Fisher scientific, USA
PS-Microplate 96 wells	Greiner Bio One International GmbH, Austria
F96 MicroWell™ Polystyrolplatt	NUNC A/S, Denmark
96 wells E-plate	ACEA Biosciences, Inc., USA
Multi-channel pipettes	Sartorius research GmbH, Germany
Cytocentrifuge	Thermo Fisher scientific, USA
Orbital shaker	Gesellschaft für Labortechnik mbH, Germany
PAP pen	Kisker Biotech GmbH & Co. KG, Germany

Slides	R. Langenbrinck GmbH, Germany
Molds for cryo-embedding	Sakura Finetek Europe, Netherlands
Cannula	B. Braun melsungen AG, Germany
Analytical balance	Sartorius research GmbH, Germany
Fluid aspiration system	Vacuubrand GmbH, Germany
Cells counter	Schärfe system GmbH, Germany
Spectrophotometer	Thermo Fisher scientific, USA
RTCA SP Analyzer	Roche, Penzberg, Germany

2.1.2 Reagents and chemicals

Name	**Company**	**Catalogue number**
Natural Product Library	TimTec, Germany	PO105733-208XLS
PVA	Merk Schuchardt OHG, Germany	4022536472362
Pancol	PAN biotech, Germany	1210916
RPMI 1640 medium	Biochrom GmbH, Germany	0227E
L-Glutamin	PAN biotech, Germany	P04-80050
Mounting medium	Merck KGaA, Germany	1.07961.0500
HEPES	Cell Concepts GmbH., Germany	celc-3958
Eosin G	Carl Roth GmbH, + Co. KG, Germany	3137.2
Ethanol	Sigma Aldrich chemie GmbH, Germany	32205
Hematoxylin Gill II	Carl Roth GmbH, + Co. KG, Germany	T864.2
DMSO	SERVA GmbH, Germany	20385.01
Luminol	Sigma-Aldrich, Germany	94H3644
Alamar Blue	AbD Serotec Ltd, UK	080909C
Diff-Quik I	Medion Diagnostics AG	661214021A
Diff-Quik II	Medion Diagnostics AG	661314021A
Diff-Quik Fix	Medion Diagnostics AG	661114021A
BSA	PAN biotech, Germany	H160811
Heparin- Natrium	B.Braun Melsungen AG, Germany	2047217
Braundest water	B.Braun Melsungen AG, Germany	190478002
Tween	Sigma.ALDRICH, Germany	9005-64-5
NaCl	Merck KGaA, Germany	1.06404.5000
KCl	Merck KGaA, Germany	1.04936.0500
Na_2HPO_4	Merck KGaA, Germany	1.06580.1000
KH_2PO_4	Merck KGaA, Germany	A434173
Ethanol	Sigma Aldrich chemie GmbH, Germany	32205
Aceton	Merck KGaA, Germany	825 K10590614
Giemsa stain solution	Sigma-aldrich international GmbH, Germany	GS1L-1L

2.1.3 Buffers and solutions

Name	Recipe
D-PBS	Braundest water: 1L NaCl: 8 g KCl: 0.2 g Na_2HPO_4: 1.15 g KH_2PO_4: 0.2 g
CL-medium	NaCl: 0.659g HEPES: 10ml RPMI1640 Medium: 500ml L-Glutamin: 5ml
Washing buffer	BSA: 5g Tween: 0.25 ml D-PBS: 500 ml
Double concentrated D-PBS	Braundest water: 1L NaCl: 16g KCl: 0.4g Na_2HPO_4: 2.88g KH_2PO_4: 0,4g

2.1.4 Antigen and Antibodies

The recombinant fragment of NC1 domain of murine COL7 and rabbit anti-murine COL7 IgG were kindly provided by Prof. Ralf J. Ludwig from the department of Dermatology, University of Lübeck, Lübeck, Germany. As described previously, the recombinant fragment of NC1 domain of murine COL7 was glutathione-S-transferase(GST)-tagged (C. Sitaru et al. 2005). The corresponding anti-COL7 IgG was generated by immunizing New Zealand White rabbits with recombinant GST-tagged COL7 fragments. Sera from immunized New Zealand White rabbits were then collected, and rabbit anti-COL7 IgG was purified from rabbit serum samples using Protein G Sepharose Fast Flow affinity column (C. Sitaru, Schmidt, et al. 2002). The binding ability and pathogenicity of the purified anti-COL7 IgG were determined with IF microscopy and *ex vivo* model, respectively (C. Sitaru, Kromminga, et al. 2002a).

2.2 Methods

2.2.1 Workflow of the current study

The overview of the workflow of the current study is shown in Figure 1. First of all, we screened a natural product library containing 800 compounds using the *in vitro* system of IC-mediated neutrophil activation to identify natural products that are capable of inhibiting neutrophil adhesion. Secondly, natural products identified in the screening were evaluated in a dose-response assessment, and only candidates that show good dose-response relationships were selected for the test of cytotoxicity. Thirdly, candidates that passed the cytotoxicity evaluation was further tested for their specificity in inhibition of neutrophil adhesion. Finally, the therapeutic efficacy of candidate compounds was determined using an ex vivo model of EBA.

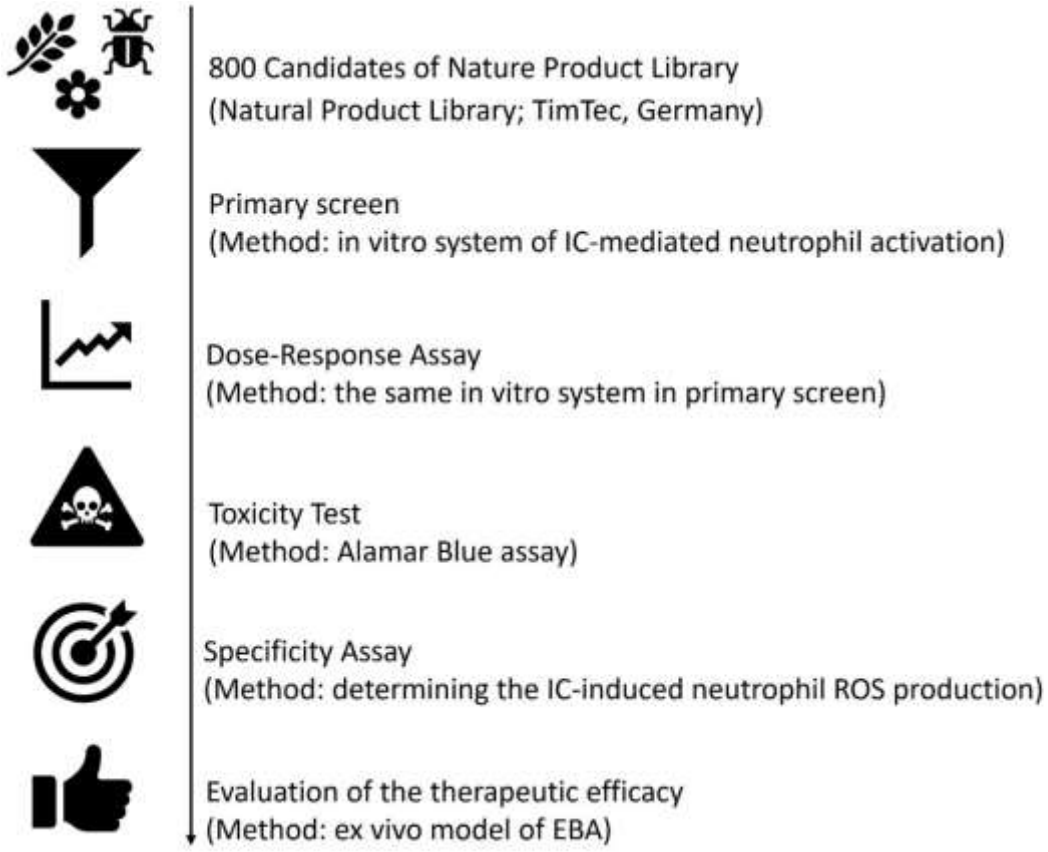


Figure 1. A schematic overview of the workflow of the current study.

2.2.2 Preparation of the Immobilized Immune Complex

Immobilized immune complexes were formed from the binding of rabbit anti-mouse COL7 IgG to recombinant mouse COL7. Wells of E-Plate (for adhesion test) or opaque (for ROS studies) 96 well plates were coated with 0.5 µg recombinant COL7 in 50 µl PBS overnight at 4°C and subsequently blocked with 200 µl 1% BSA with ultra-low endotoxin at 37 °C for 2 hours. After incubation with 50 µg rabbit anti-mouse COL7 in 50 µl 1% BSA containing 0.05% Tween-20 at 37 °C for 2 hours, plates were finally washed with CL-medium. Control wells received either medium alone instead of murine COL7 or rabbit anti-mouse COL7 IgG was replaced by normal rabbit control IgG.

2.2.3 Isolation of Neutrophils

Neutrophils were isolated from peripheral blood of healthy donors by sedimentation and density gradient centrifugation as previously described (B. Kasper et al. 2004; B. Kasper et al. 1997). This study was performed in accordance with the 1964 Helsinki Declaration, and the approval was obtained from the institutional ethics committee of the University of Lübeck (Number: Az 12-202A, from April 25, 2019). Briefly, peripheral blood was collected in a sterile tube containing the anticoagulant heparin (20U/ml). Then the heparin-treated blood was gently mixed with the same volume 1% PVA, and the solution was placed at room temperature for 30 minutes. The supernatants of the blood sample solution were gently transferred into a new tube containing 10 ml Pancol. Then samples were centrifuged at 300 g for 24 minutes at room temperature, and the supernatant, as well as the cloudy band of mononuclear cells, were aspirated carefully. Thereafter, residual red blood cells in the cell pellet were lysed by ice-cold Braundest H2O for 45 seconds, and the lysis was stopped by adding the same volume of double-concentrated D-PBS. Next, the neutrophil suspension was centrifuged and washed three times with cold D-PBS and resuspended in CL-medium (1×10^6 cells/mL; RPMI 1640 buffered with 25 mM HEPES without phenol red; Biochrom, Berlin, Germany) with 1% L-glutamine. Finally, neutrophils were counted and suspended in CL-medium with 1% L-glutamine at desired concentrations.

2.2.4 Determination of Viability and Purity of Neutrophils

Viability of neutrophils was determined with Trypan Blue exclusion methods as described previously (Strober 2001). Briefly, 10 µl neutrophil suspension at the concentration varying from 10^5 to 10^6 per ml were mixed in suspension with the same volume of Trypan Blue solution. After 3 minutes incubation, viable and dead cells were counted using a hemocytometer. Cell viability was calculated as the percentage of viable cells in total neutrophils.

The purity of neutrophils was determined by the morphology of stained cytospins. For cytospin, 1×10^5 neutrophils in CL medium were centrifuged onto a glass slide at 55 g for 7 min using a Cytocentrifuge and immediately stained with Diff-Quik Staining set according to the provided protocol. Briefly, cytospin slides were incubated with Diff-Quick Fix solution for 5 × 1 second, followed by incubation

with Diff-Quik I and II solutions for 6 × 1 second and 4 × 1 second, respectively. Finally, slides were washed with D-PBS and then evaluated under the microscope.

2.2.5 Measurement of IC-induced Neutrophil Adhesion

IC-induced neutrophil adhesion was measured by an electronic impedance-based assay using the xCELLigence RTCA instrument which allows a real-time monitoring of the adhesion properties of cells *in vitro* in a non-invasive label-free manner (Scrace et al. 2013; Bird and Kirstein 2009). The xCELLigence RTCA system is composed of an electronic impedance detector named RTCA SP Analyzer, a computer equipped with RTCA Software, a 96-well E-Plate incorporated with sensor gold electrode array and a cell culture incubator. During the measurement, the electronic readout of cell-sensor impedance was determined in real-time converted into cell index (CI) (CI) using the following formula: CI(t)= R(fn,t)-R(fn,t0) /Zn, where R(fn,t) is the measured impedance at frequency fn at time point t and R(fn,t0) is the measured impedance at frequency fn at time point t0 ("Calculation Principles of RTCA Software," n.d.).

To determine the IC-induced neutrophil adhesion, immobilized IC was generated on wells of E-plate using the protocol described in 2.2.2. Then freshly isolated human neutrophils were added to the plate, and the real-time cell index of each well was detected every minute for a total measurement period of 90 minutes. Cell indexes were converted to Delta Cell Indexes by setting the starting time point as the Delta time and plotted in the Plot tab. The area under the curve (AUC) of the cell index curve was calculated and used as the index of the neutrophil adhesion.

2.2.6 Dose-Response Assay

A dose-response assay was performed to evaluate the inhibitory effect of candidate compounds at various concentrations on IC-induced neutrophil adhesion using the xCELLigence RTCA system. Briefly, immobilized IC was prepared on wells of E-plate and freshly prepared neutrophils were added to wells in presence of various concentrations of candidate compounds, including 10 μM, 1 μM, 0.1μM and 0 μM. Cell indexes were recorded for 90 minutes, and AUC of Delta Cell Indexes was calculated. For further analysis, neutrophil adhesion was expressed as relative adhesion index where the value of AUC of positive control (neutrophils stimulated with IC in absence of the compound) and negative control (unstimulated neutrophils) were set as 100% and 0%, respectively.

2.2.7 Cytotoxicity Test

The cytotoxicity of candidate compounds was determined by utilizing the Alamar Blue assay. Freshly isolated neutrophils at a final concentration of $1x10^6$/ml with a total volume of 150 µl in CL-Medium were stimulated with immobilized IC in presence or absence of candidate compounds at a concerntration of 1 µM (A) or 10 µM (B) at 37°C for 2 hours. Subsequently, 15 µl Alamar Blue reagent was added to each well, and spectrophotometric absorbance was determined every 60 mins for 7 hours at two wavelengths: 570 nm and 600 nm. Cell viability was expressed as a relative value where the value of positive control (unstimulated neutrophils) was set as 100%.

2.2.8 Measurement of ROS Production from Neutrophils

To determinate IC-induced neutrophil ROS production, immobilized COL7-anti-COL7 IgG IC was prepared on solid opaque 96-well plates using the method described in 2.2.2. Freshly isolated neutrophils were suspended in 2×10^6/ml in CL medium supplemented with 5 µM luminol (5-Amino-2, 3-dihydro-1, 4-phthalazinedione; Roche Applied Science) and distributed in 200 µl aliquots in an untransparent 96 well microtiter plate coated with IC or with corresponding controls. After addition of neutrophils onto plates with immobilized immune complex, chemiluminescence was recorded by microplate luminometer for 60 min in a real-time manner and data were expressed as relative light units (RLU).

2.2.9 Ex vivo Model of EBA

The ex vivo model of EBA was performed as previously described with slight modification (C. Sitaru, Kromminga, et al. 2002b). Firstly, 6 µm cryosections prepared from the tail skin of C57BL/6 mice were defrosted at room temperature, washed with PBS for 5 minutes to remove embedding medium, and then incubated with 0.2 mg/ml rabbit anti-mouse COL7 IgG for 2 hours at 37°C in a humidified air incubator containing 5% CO_2. After washing the sections with PBS twice, chambers were prepared as described and 500 µl of freshly prepared human neutrophil suspension at a concentration of 3×10^7 cells/ml in CL medium supplemented with 10% human serum was placed in each chamber (C. Sitaru, Kromminga, et al. 2002b). To study the inhibition effect of natural products, neutrophils were incubated with candidate compounds at a final concentration of 10 µMat 37°C for 5 min before they were transferred into the chambers. Incubation of neutrophils with skin sections was performed in a humidified air incubator containing 5% CO_2 for 3 hours at 37°C. Subsequently, chambers were disassembled; sections were washed in PBS, fixed with acetone for 10 minutes, and stained with hematoxylin and eosin. Skin dermal-epidermal separation was evaluated by light microscopy, and the extent of dermal-epidermal separation was analyzed in a blinded fashion by two persons.

2.2.10 Statistical Analysis

All data are expressed as the mean ± SD (standard deviation) or mean ± SEM (standard error of mean). Quantative data was firstly examined with Kolmogorov-Smirnov normality test. For the normally distributed data, statistical difference was determined by two-tailed paired Student's t test, otherwise Wilcoxon signed rank test was used. For the data with two or more independent varaibles, statistical analysis was performed with two-way ANOVA test. For the evaluation of dose-response relationship, linear regression analysis was performed, and the F-test was applied to assess the statistical significance. P values less than 0.05 were considered as statistically significant.

3 Results

3.1 Primary screening

To identify potential candidate compounds that are able to inhibit IC-induced neutrophil adhesion, I screened the Natural Products Library which contains 800 compounds. The primary screening was conducted with an *in vitro* neutrophil activation system where neutrophils adhesion induced by the COL7-anti-COL7 IgG IC was quantified by using the xCELLigence RTCA system in a real-time manner for 90 min. All 800 natural products were tested at the concentration of 5 μM in two independent experiments with neutrophils from two healthy donors. Neutrophil adhesion was quantified and expressed as the relative AUC where the values of AUC of positive control (neutrophils stimulated with IC in absence of a compound) and negative control (unstimulated neutrophil) were set as 100% and 0%, respectively.

Among the 800 tested natural products, 11 showed relative AUC values of lower than 50% (Figure 2). According to their positions in the library, these 11 natural products were encoded as P1D10, P1G10, P4F8, P5H10, P6G3, P6F5, P6E6, P7G5, P8H8, P10B6, and P10H4. They represent luteolin peracetate, luteolin, capsaicin, gossypol, gossypolone, digitonin, plumbagin, estradiol valerate, 2-Chloroadenosine, 3,3-Diindolylmethane, and peracetate rutine, respectively.

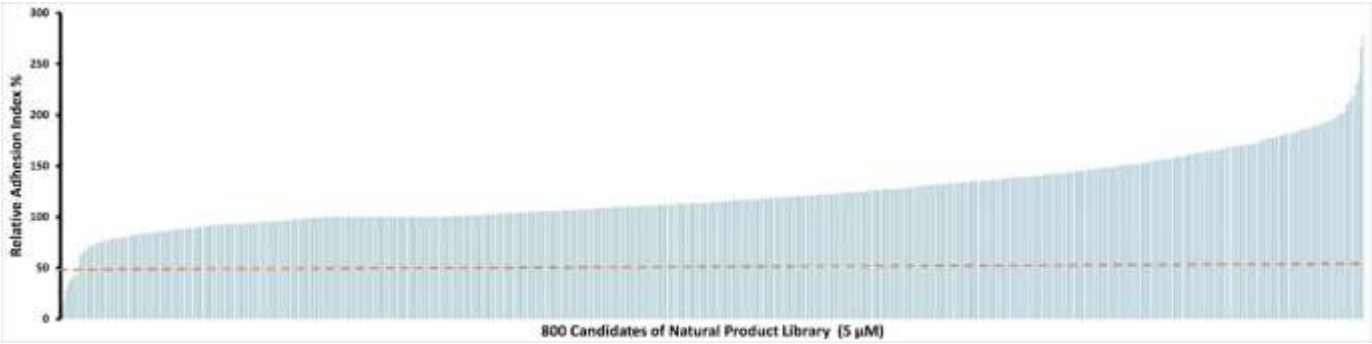


Figure 2. Effects of 800 natural products on IC-mediated neutrophil adhesion.

Freshly isolated human neutrophils (2×10^6/ml) were stimulated with immobilized COL7-anti-COL7 IgG IC in the presence or absence of a natural product at a concentration of 5 μM. Neutrophil adhesion was determined by real-time impedance measurement for 90 minutes using xCELLigence RTCA and expressed as relative adhesion index where the value of AUC of positive control (neutrophils stimulated with IC in absence of the compound) and negative control (unstimulated neutrophils) were set as 100% and 0%, respectively. The X-axis stands for the 800 compounds of natural product library, and the Y-axis presents the relative adhesion index. The dashed line represents 50% adhesion index of the positive control.

3.2 Dose-response relationships of 11 natural products

After the primary screening, 11 natural products which showed an inhibitory effect on IC-mediated neutrophil adhesion were selected for further evaluation. Dose-response relationships of drugs not only help to predict the efficacy and potency of therapeutic candidates but also show benefit for the interpretation of the interaction between drugs and their targets (J. E. Campbell and Cohall 2017). In this part, I determined the dose-response relationships of the 11 natural products by detecting the inhibitory effect of them on IC-mediated neutrophil adhesion at four concentrations, namely 10 μM, 1 μM, 0.1 μM and 0 μM. The dose-response assay was performed in four independent experiments by using the xCELLigence RTCA system with neutrophils isolated from 4 healthy blood donors, respectively.

Among the 11 tested natural products, 5 products, namely luteolin peracetate, plumbagin, digitonin, gossypol, and gossypolone showed significant dose-response relationships (Figure 3). Therefore, these five compounds were selected for further evaluation.

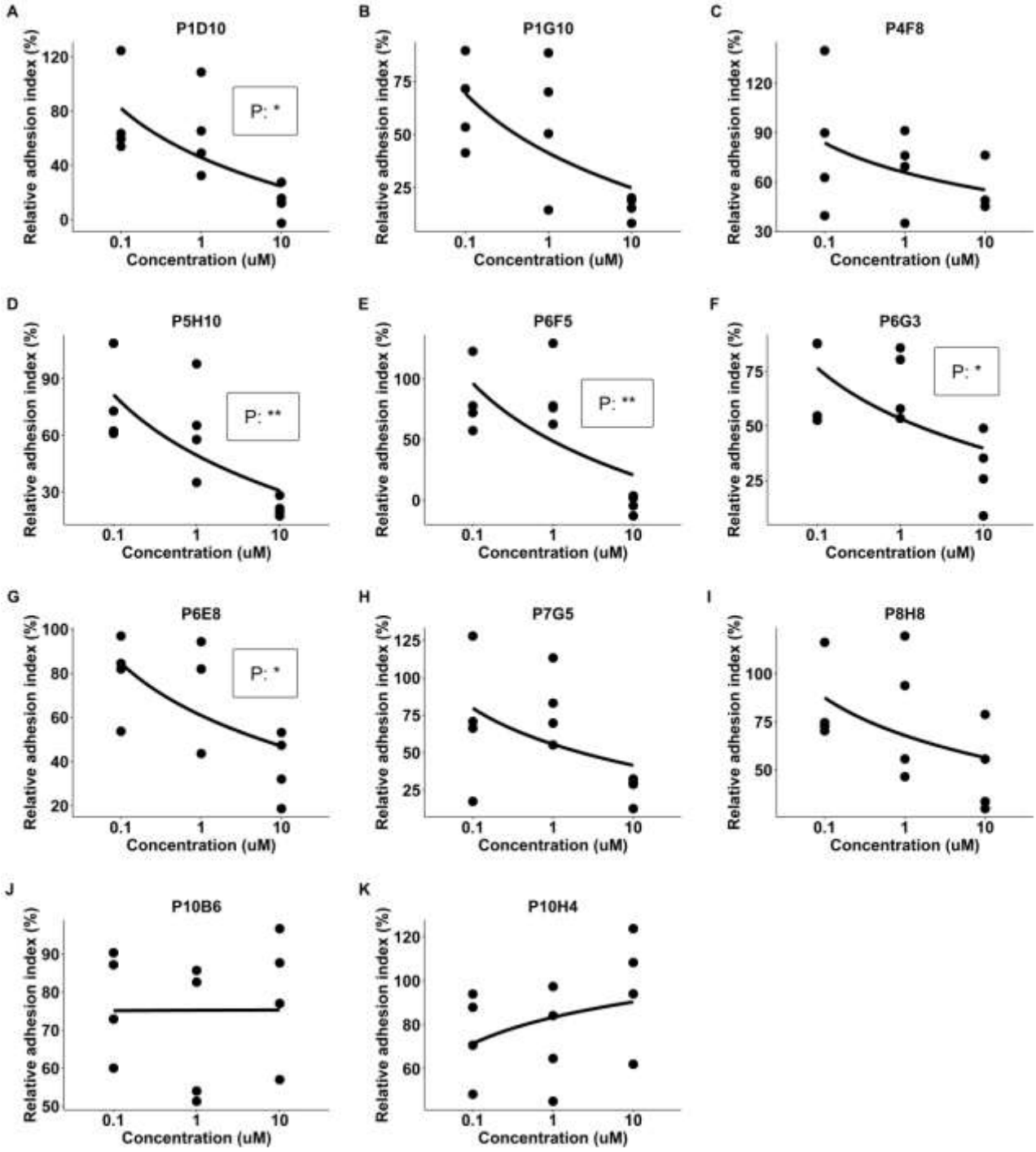


Figure 3. Dose-response relationships of 11 natural products.

Freshly isolated neutrophils were stimulated with immobilized immune complex (IC) on wells of E-plate in presence of indicated concentrations of candidate compounds, including Luteolin peracetate (A), Luteolin (B), Capsaicin (C), Gossypol (D), Gossypolone (E), Digitonin (F), Plumbagin (G), estradiol valerate (H), 2-Chloroadenosine (I), 3,3-Diindolylmethane (J), and Peracetate Rutine (K). Cell indexes were determinedrecorded for 90 minutes, and AUC of Delta Cell Indexes was calculated. For further analysis, neutrophil adhesion was expressed as relative adhesion index where the value of AUC of positive control (neutrophils stimulated with IC in absence of the compound) and negative control (unstimulated neutrophils) were set as 100% and 0%,

respectively. The X-axis presents doses of natural products, while Y-axis shows the relative adhesion indexes. Data from four independent experiments were used for analysis. Linear regression analysis was performed to evaluate the dose-response relationship, and the statistical significance was assessed using the F-test (*$p<0.05$, **$p<0.01$).

3.3 Cell toxicity

To exclude that inhibition on neutrophil adhesion is a consequence of toxic effects, I performed the toxicity test for the five natural products. The toxicity was accessed by determining the cell viability using the Alamar Blue cytotoxicity assay. Neutrophils were stimulated with immobilized IC in the presence or absence of candidate natural products at a concentration of 1 μM or 10 μM, and Alarm Blue was added to cells 1 hour later to monitor the cell viability for 6 hours.

At the concentration of 1 μM, plumbagin showed significant and dramatic toxic effects on neutrophils, with a decrease of 50 percent in cell viability (Figure 4A). At the concentration of 10 μM, significant toxic effects were observed for both plumbagin and digitonin, with decreases of 70 percent in cell viability for both products (Figure 4B). In addition, Gossypolone at the concentration of 10 μM also showed a significant but mild toxic effect on neutrophils, with a decrease of roughly 20 percent in cell viability, but such effect was not observed at the concentration of 1 μM (Figure 4). Therefore, luteolin peracetate, gossypol, and gossypolone were selected for further investigation.

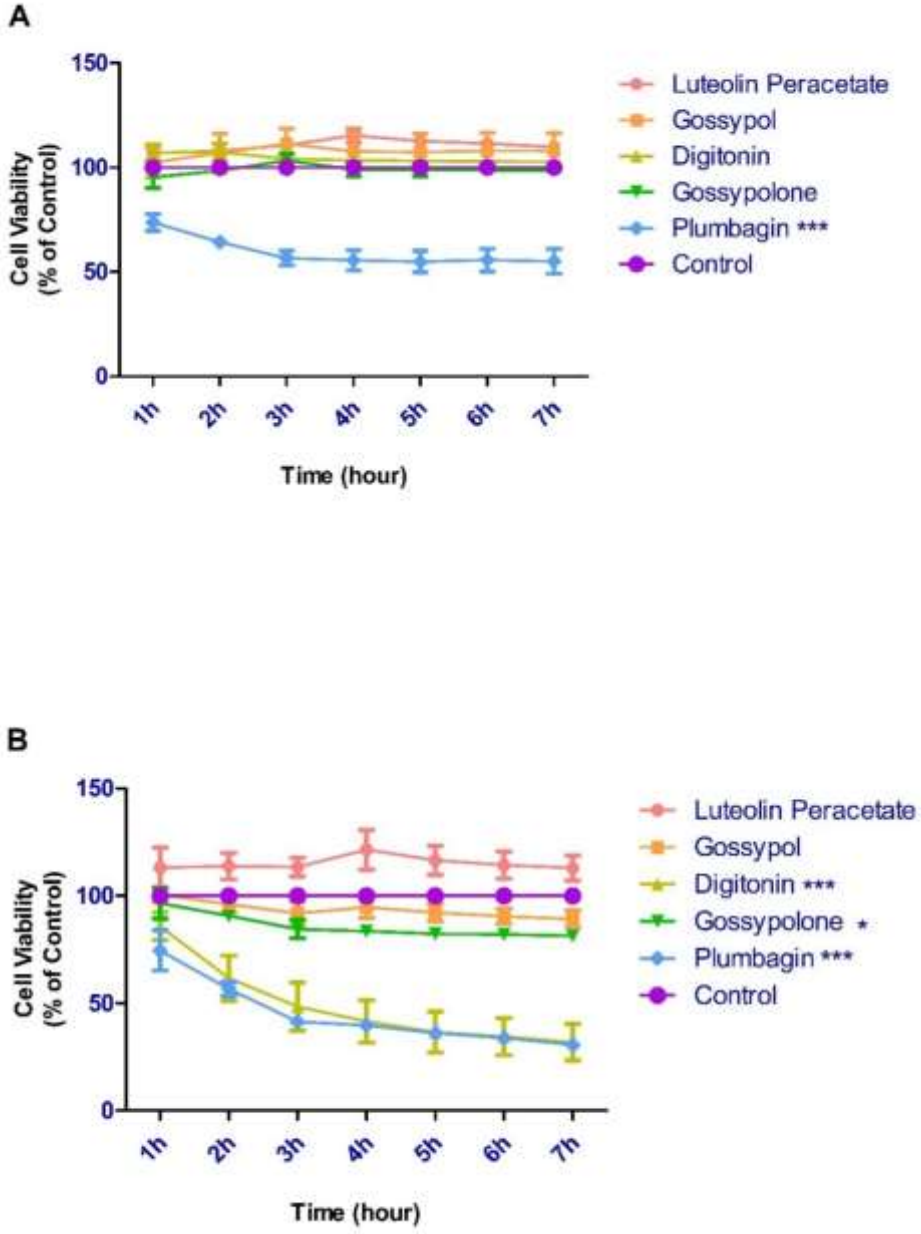


Figure 4. Toxic effects of 5 natural products on neutrophils.

Freshly isolated human peripheral blood neutrophils were stimulated by immobilized IC in presence or absence (control) of indicated natural product at a concentration of 1 μM (A) or 10 μM (B). Cell viability was assessed by using the Alamar Blue cytotoxicity assay. The Alarmar Blue was added 1 hour after the cell incubation, and the absorbance was recorded every hour for 6 hours. Data are presented as mean ± SEM (standard error of the mean) of 4 independent experiments. Cell viability was expressed as a relative value where the value of control (unstimulated neutrophils) was set as 100%. The toxic effect of each natural product was evaluated by comparing the cell viability between product-treated neutrophils and controls using two-way ANOVA test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

3.4 Effect of selected natural products on IC-mediated neutrophil ROS production

Upon stimulation with immobilized IC, activated neutrophils not only adhere to the IC-coated surface but also release granule proteins and generate ROS (Yu et al. 2018). Therefore, in the next step I investigated whether the three selected candidates could specifically inhibit the neutrophil adhesion without affecting other biological events of IC-induced neutrophil activation. Using a chemiluminescence assay, I determined the effect of the three selected natural products, luteolin peracetate, gossypol, and gossypolone, on IC-induced ROS production from neutrophils.

To figure out the effects of the 3 selected candidates on ROS production of neutrophil, freshly isolated neutrophils were stimulated with immobilized IC (coated mCOL7 incubated with rabbit anti-mCOL7 IgG) or control (coated mCOL7 incubated with normal rabbit IgG) in presence or absence of the respective candidate compounds at a concentration of 10 µM. As shown in Figure 5A, time kinetics of ROS production showed that neutrophils immediately produced ROS when they attached to immobilized IC. The produced ROS reached a peak at approximately 10 minutes and returned to background level after 40 minutes. In contrast, neutrophils exposed to control surfaces did not show a significant ROS production. When neutrophils were exposed to immobilized IC in the presence of luteolin peracetate, gossypol or gossypolone, the IC-mediated ROS production was significantly decreased. Quantification of the ROS production in 6 experiments showed that all three natural products significantly inhibited the IC-induced ROS production from neutrophil (Figure 5 B).

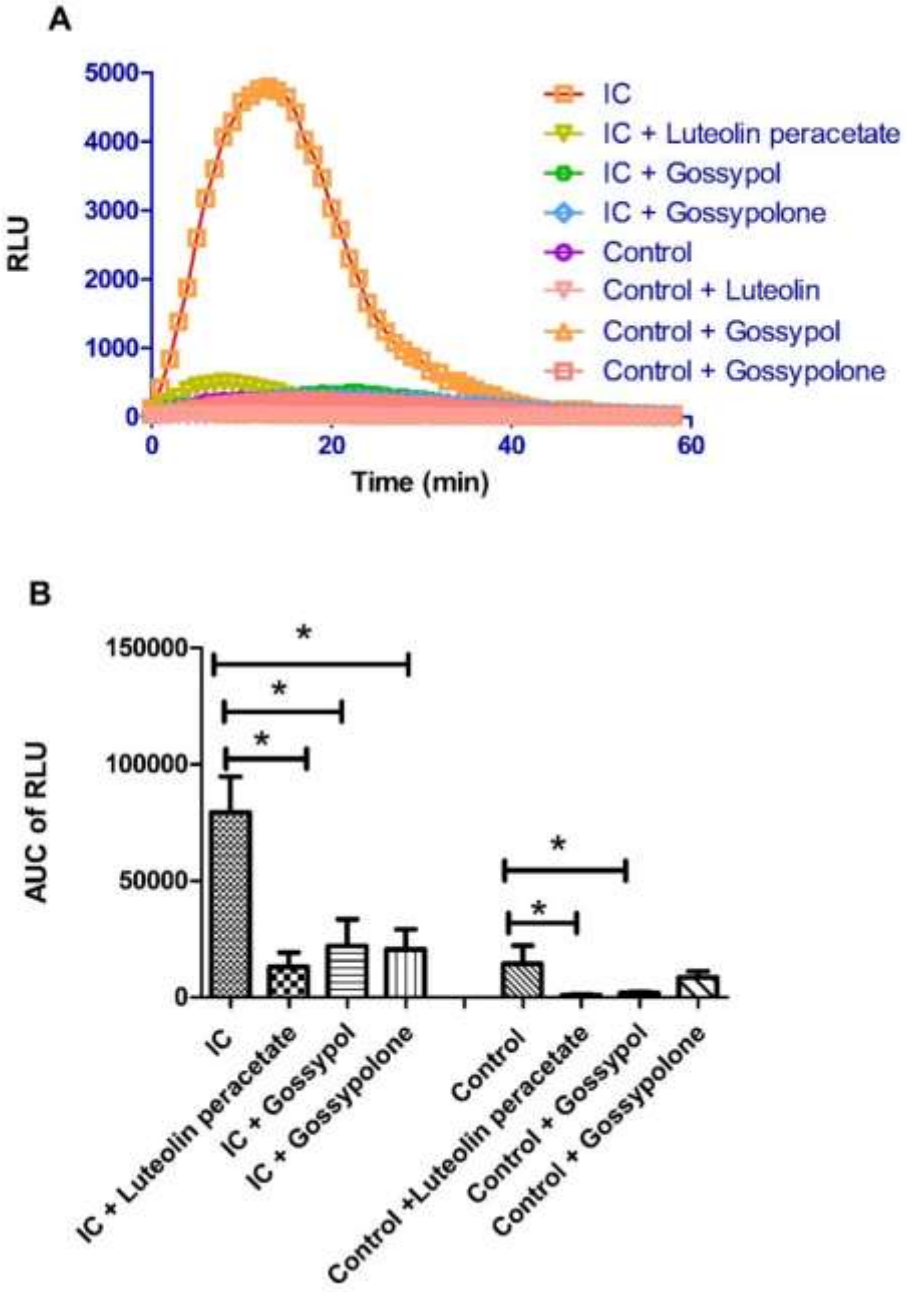


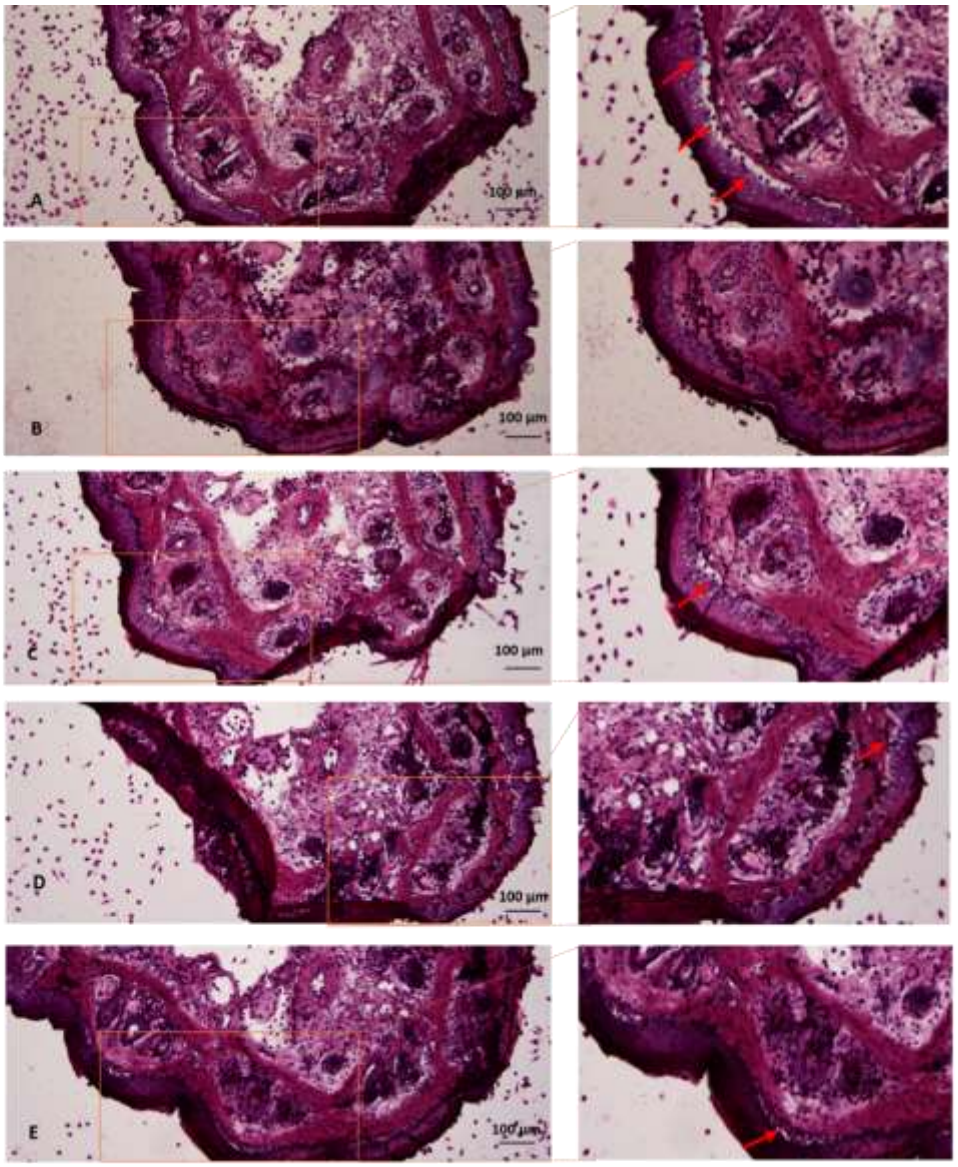
Figure 5. Effect of selected natural products on IC-induced ROS production of neutrophils.

Freshly neutrophils were stimulated with immobilized IC or control in the presence or absence of a natural product at the concentration of 10 μM. Generation of ROS was determined by recording chemiluminescence using microplate luminometer for 60 min in a real-time manner and data were expressed as relative light units (RLU). A. Representative kinetics of ROS production from neutrophils under indicated experimental conditions. B. Kinetic data of IC-mediated ROS production from neutrophils were integrated and represented as the AUC (area under the curve) of RLU (relative light units). Data are presented as mean ± SD derived from 6 experiments, and statistical analysis was performed using Wilcoxon signed rank test (*$p<0.05$).

3.5 Effect of selected natural products in experimental EBA *ex vivo*

Finally, I determined whether the three natural products, luteolin peracetate, gossypol, and gossypolone can inhibit the neutrophil-causes tissue damage in an ex vivo model of EBA. In this *ex vivo* model, cryosections of mouse tail skin were incubated with rabbit anti-mouse COL7 IgG followed by the incubation with freshly isolated human neutrophils in the presence or absence of an inhibitor (Yu et al. 2014). Thereafter, the cryosections were fixed and then stained with hematoxylin and eosin, and skin separation was quantified as the percentage of the length of epidermis detachment in relation to the length of the total dermal-epidermal zone.

In this model, neutrophils activated by the COL7-anti-COL7 IC caused the dermal-epidermal separation of 33±7.9% while no separation was observed in the negative control (Figure 6). In the presence of 10 μM luteolin peracetate, the dermal-epidermal separation was significantly reduced to 4.9±2.6% (P = 0.009). Significant and dramatic reduction in dermal-epidermal separation was also observed in the samples treated with 10 μM gossypolone (16.1±8.4%, *P*=0.0091). In the presence of 10 μM gossypol, although the dermal-epidermal separation was also reduced to 12.5±8.6%, this difference was not significant (*P*=0.0547). Taken together, these results suggest that both luteolin peracetate and gossypolone are able to inhibit tissue damage in the ex vivo model for EBA. However, the group of gossypol showed no significantly different between the positive control group.


A
100 µm
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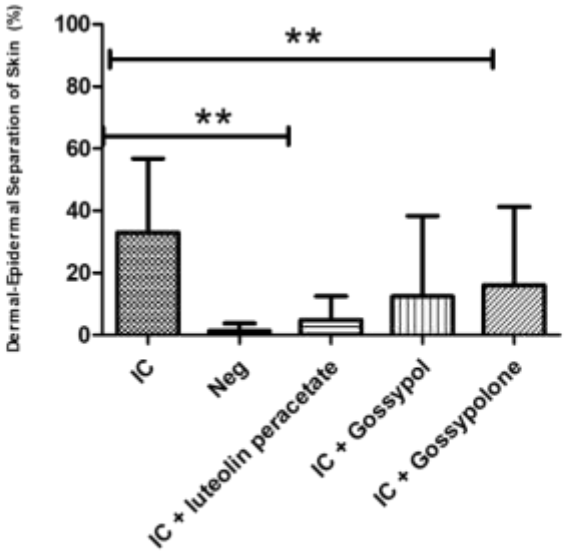
Dermal-Epidermal Separation of Skin (%)
100
80
60
40
20
0
**
**
IC
Neg
IC + luteolin peracetate
IC + Gossypol
IC + Gossypolone

F

Figure 6. Effect of luteolin peracetate , gossypol and gossypolone on tissue damage in experimental EBA *ex vivo*.

Mouse skin cryosections were incubated with 0.2 mg/mL rabbit control IgG (**B**) or rabbit-anti-mouse COL7 IgG (**A, C-E**) for 1 hour at 37°C. Subsequently, specimens were exposed to freshly isolated human neutrophils (**A, B**) or neutrophils in presence of 10 µM luteolin peracetate (C), 10 µM gossypol (D) or 10 µM gossypolone (E). Images of the skin section were acquired using the NIS-Element D 3.0 software and an OLYMPUS Bx41 microscope. Micrographs of a representative experiment are shown, and arrows indicate the dermal-epidermal separation. Scale bar = 100 µm. Skin separation was quantified as percentage of the length of epidermis detachment in relation to the length of the total dermal-epidermal zone (F). Data are presented as mean ± SD of 9 independent experiments. Statically significant differences were performed using Wilcoxon signed rank test (*P<0.05, **P<0.01).

4 Discussion

EBA is a rare autoimmune disease, which is characterized by dermal-epidermal separation mediated by the autoantibodies against COL7. In the inflammatory form of EBA, neutrophils play an essential role in the tissue damage of the disease, and neutrophil adhesion has been shown to be an indispensable step in the process. In the current study, I screened a natural product library to identify chemical compounds that are able to inhibit IC-mediated neutrophil adhesion and evaluated their efficacy in modulating tissue damage in an *ex vivo* model of EBA.

In the first part of the current study, a primary screening was carried out for all 800 compounds using an in vitro assay system, in which inhibitory effects of each compound on IC-induced neutrophil adhesion was detected in a real-time approach. Eleven compounds selected from the primary screening were subjected to a dose-response assay, of which 5 of them, namely luteolin peracetate, gossypol, digitonin, gossypolone, and plumbagin, showed a significant dose-response relationships and were further evaluated. By determining the cytotoxic side effects on neutrophils of the 5 selected compounds, luteolin peracetate, gossypol and gossypolone were selected for final evaluation for their therapeutic efficacy. Finally, two natural productions, luteolin peracetate and gossypolone, showed a strong and significant inhibitory effect on IC-induced neutrophil tissue damage in an *ex vivo* model for EBA.

Copmpared with conventional synthetic molecules, natural products (NPs) are traditionally valued source of potential therapeutic compounds in drug discovery and development (Atanasov et al. 2015; Harvey, Edrada-Ebel, and Quinn 2015). However, since 1990s, the trend of drug discovery research has shifted to synthetic compounds owing to the high effectiveness of high-throughput screening (HTS) and the several technical limits with NPs such asisolation, characterization and optimization of bioactive compounds (Henrich and Beutler 2013; Atanasov et al. 2015). Besides, due to intellectual property rights hurdle and regulations issues of biological material, it's also challenging for pharmaceutical companies to commercialize the identified NPs as drugs in market (Harrison 2014; Burton and Evans-Illidge 2014; Heffernan 2020). With recent technological and scientific advances in analytical techniques, genome mining and engineering as well as cultivation systems, the abovementioned technical challenges of NP-based drug discovery may be properly handled, indicating that NPs are still a promising source for exploring potential therapeutics (Atanasov et al. 2021; Moffat et al. 2017). In this study, I screened 800 NP-derived compounds and identified 2 potential candidates as therapeutics for the treatment of EBA. Therefore, the current study provides a good example of exploring the therapeutic potential of natural products.

In total, 11 of 800 compounds which passed the primary screening were further tested for their dose-response relationships. Five of them, namely luteolin peracetate, plumbagin, digitonin, gossypol, and

gossypolone showed significant dose dependency in the inhibition of IC-induced adhesion. Given that dose-response relationships of drugs not only help to predict the efficacy and potency of therapeutic candidates (J. E. Campbell and Cohall 2017), these five natural compounds are of our special interest and thus selected for further evaluation.

Notably, two natural products, plumbagin and digitonin, showed toxicity effect to neutrophils. Although it has not been reported whether plumbagin is toxic for neutrophils, its toxic effect on tumour cells has been well documented because it is suggested as a promising anti-cancer compound (Tripathi, Panda, and Biswal 2019; Sumsakul, Plengsuriyakarn, and Na-Bangchang 2016). For example, it has been shown that plumbagin is able to inhibit the growth of MCF-7 (breast adenocarcinoma) and BJ (normal skin fibroblasts) cell lines in a dose- and time-dependent manner (Sagar et al. 2014). The cytotoxicity effect of digitonin, a non-ionic detergent, has also been well demonstrated (Z. Wang et al. 2007). Furthermore, it has been shown that digitonin can decrease the viability of neutrophils via permeabilizing cells and influencing lysosomal enzyme release (Sandborg and Smolen 1989; Smolen, Stoehr, and Boxer 1986).

Two compounds, luteolin peracetate and gossypolone, showed a strong and significant inhibitory effect on IC-induced neutrophil tissue damage. Interestingly, both luteolin peracetate and gossypolone did not only inhibit the IC-mediated neutrophil adhesion, but also blocked IC-mediated ROS production of neutrophils. These findings suggest that both natural products represent inhibitors for neutrophil activation in general.

Luteolin peracetate is a flavonoid. According to the typical structures with C6-C3-C6, there are more than six thousands compounds have been classified as flavonoids (Panche, Diwan, and Chandra 2016). Although it is well established that flavonoids are featured by pharmacological properties of anti-inflammatory, immunomodulatory functions, antioxidant, anti-cancer and antimicrobial effects (Lalani and Poh 2020; Nabavi et al. 2015), to my best knowledge the pharmacological property of luteolin peracetate remain unknown. However, luteolin, a flavonoid highly related to luteolin peracetate, has been well studied. Luteolin is one of flavones, which is classified as flavonoids based on the backbone of 2-phenyl chromen-4-one (López-Lázaro 2009). As one of the most common flavonoids, luteolin are found in around 300 plant species, like malvaceae, asteraceae, lamiaceae et al. (López-Lázaro 2009). luteolin has been also identified in leaves of many edible plants, such as celery, broccoli, green pepper, parsley, thyme, dandelion, perilla, chamomile tea, carrots, olive oil, peppermint, rosemary, navel oranges, and oregano (López-Lázaro 2009; Shimoi et al. 1998).

The current study showed that luteolin is able to inhibit neutrophil adhesion induced by immobilized IC. It has been reported that flavonoids inhibit neutrophils migration both *in vitro* and *in vivo* by acting on

multiple mechanisms, including decreasing the expression of β2-integrin expression of neutrophils (Suyenaga et al. 2014). In addition, it has been demonstrated that the IC-induced neutrophil adhesion is dependent on β2-integrin (Yu et al. 2018). Therefore, luteolin might inhibit the IC-induced neutrophils adhesion by inhibiting the β2-integrin expression on neutrophils. However, this hypothesis needs to be validated in future studies.

Upon stimulation of immobilized IC, neutrophils not only adhere to the IC-coupled surface, but also generate ROS and release granule proteins. Interestingly, luteolin also inhibits the IC-mediated ROS production from neutrophils. An inhibitory effect of luteolin on ROS production from activated neutrophils has been reported by several studies. Around 30 years ago, 'T Hart and colleagues reported that luteolin and other 3 flavonoids are able to inhibit the $O2^{-}$-release from activated human neutrophils by determining the luminol-dependent chemiluminescence (T Hart et al. 1990). Apart from the suppression of O2- release, luteolin also shows inhibitory effects on the generation of superoxide anion from fMLP-stimulated human neutrophils (H. W. Lu et al. 2001). Moreover, Oswald et al reported that luteolin is able to suppress the ROS production from neutrophils stimulated with COL17-anti-COL17 IC in a dose-dependent manner (Oswald et al. 2012), which is in line with our findings. Therefore, luteolin is capable of inhibiting ROS production from neutrophil activated by multiple stimuli. The mechanism behind the inhibitory effect of luteolin or other flavonoids on neutrophil ROS production has been studied extensively. In 1995, Belyakov et al. for the first time reported that the OH-groups located in the B-ring of the luteolin plays an essential role in the inhibition of neutrophil ROS production (Belyakov, Roginsky, and Bors 1995). By systematically comparing the different flavonoids using trolox equivalent antioxidant capacity (TEAC) assay, Rice-Evans et al identified two feature structures of luteolin showing antioxidant effects, namely, an ortho-dihydroxy structure in the B-ring and a 2,3-double bond in conjunction with the 4-oxo function of the C-ring (Rice-Evans, Miller, and Paganga 1996). Thus, it is conceivable that luteolin inhibits immobilized IC-induced ROS production from neutrophils via certain feature structures.

Notably, luteolin peracetate at the concentration of 10 μM significantly inhibited the tissue damage in the ex vivo model of EBA. In traditional Chinese medicine, the flavonoids-rich plants, such as chrysanthemum flowers, celery and peppers, have been used as Chinese traditional therapy against hypertension, inflammatory diseases, and cancer (Harborne and Williams 2000). As one of the most intensively studied flavonoids, luteolin was suggested to be one promising new therapy against various inflammatory diseases, immunological disorders and cancer (Seelinger, Merfort, and Schempp 2008; Imran et al. 2019). For example, with LPS-induced acute lung injury mice model, Kuo et al. reported the secretion of tumor necrosis factor-alpha, keratinocyte-derived chemokine, intercellular adhesion molecule-1 in the BALF and the activity of catalase and superoxide dismutase in the lung of a LPS-induced acute lung injury mouse model were inhibited by luteolin (M.-Y. Kuo et al. 2011). Given that

luteolin inhibits adhesion and ROS production from neutrophils activated by not only IC but also other stimuli, it is conceivable that luteolin is able to suppress neutrophil-mediated inflammation in general. Indeed, both in vitro and in vivo studies have already demonstrated the anti-inflammatory property of luteolin (H. P. Kim et al. 2004; C.-Y. Chen et al. 2007; Törmäkangas et al. 2005; J. S. Kim and Jobin 2005; Ziyan et al. 2007). Taken together, these findings suggest luteolin as a novel potential therapeutic for EBA, which needs to be further evaluated.

With regards to the molecule mechanisms underlying the anti-inflammation effect of luteolin, many other biological events have been suggested in addition to inhibition of neutrophil adhesion and ROS production. First of all, several in vitro studies have shown that many inflammatory cytokines and chemokines can be inhibited by luteolin in a dose dependent manner (Griffith, Sokol, and Luster 2014; Kang et al. 2010; Jeon et al. 2014). Second, by determining NF-κB transcriptional activity and the expression of IκB-α, Jia et al. reported that luteolin inhibits inflammation by suspension of NF-κB signaling pathway (Jia et al. 2015), and this finding was confirmed by other studies using various experimental models of inflammation (Weng et al. 2014; Wölfle et al. 2011). Third, Kong et al. demonstrated that the suspended cAMP-phosphodiesterases activity and expression of vascular cell adhesion molecule and soluble intercellular adhesion molecule-1 could be the potential mechanism of the anti-inflammatory effects of luteolin (Kong et al. 2019). Taken together, luteolin might exerts its anti-inflammatory effect via multiple mechanisms, including decreasing of ROS production, suppressing leukocyte adhesion, inhibiting the NFκB pathway and regulating inflammatory mediators (Seelinger, Merfort, and Schempp 2008).

In the current study, I showed that both gossypol and gossypolone were able to inhibit IC-mediated neutrophil adhesion and ROS production, without significant toxic effect on neutrophils. Furthermore, gossypolone was able to significantly inhibit the tissue damage in an *ex vivo* model for EBA. As a natural occurring polyphenol, gossypol is isolated from the seed, roots, and stem of cotton (Zeng et al. 2019). Gossypol has not only long been known as a male contraceptive because of its antifertility activity, but also been reported as a potential drug for several cancer and chronic infectious diseases owing to the recently uncovered biological effects such as anticancer, antivirus, antiparasitic and antimicrobial activities (Keshmiri-Neghab and Goliaei 2014; Zeng et al. 2019; Amini and Kamkar 2005). As a major metabolite of gossypol, gossypolone shares similar biological properties as gossypol, such as anticancer and antimicrobial effects (Mellon et al. 2014; Y. Lu et al. 2017; Lan et al. 2018). Although gossypol and its derivatives have been already extensively investigated regarding their antifertility and anticancer activities (Keshmiri-Neghab and Goliaei 2014), their anti-inflammatory activity has been largely underestimated.

It has been reported that gossypol and gossypolone show antioxidant activity and have been suggested as potential therapeutics for psoriasis (Dodou et al. 2005). Beside this, the antioxidant effects have been

suggested as a potential mechanism behind therapeutic properties of gossypol and its derivatives in cancer (Kovacic 2003). In addition, Wang and colleagues showed that the hydroxyl groups of gossypol play a significant role in scavenging free radicals in various tumor cell lines (X. Wang et al. 2008). The effect of gossypol and its derivatives on ROS production has been studied not only with tumor cell lines, but was also demonstrated for human neutrophils. For example, Benhaim et al reported that pretratment of gossypol could induce the superoxide production of human neutrophils in a time- and concentration-dependent manner, suggesting a pro-oxidant activity of gossypol (Benhaim et al. 1994). In another study, Barba-Barajas et al. reported that gossypol-induced neutrophil apoptosis could be inhibited by antioxidants, which also suggests a pro-oxidant effect of gossypol on neutrophils during the apoptosis (Barba-Barajas et al. 2009). By contrast, the current study demonstrated that both gossypol and gossypolone show a strong antioxidant effect on neutrophils stimulated with immobilized IC. Therefore, the effect of gossypol and its derivatives on neutrophil ROS production might be condition-dependent, where they show pro-oxidant effects on unstimulated neutrophils and antioxidant effects on neutrophil activated by other stimuli.

Compared to luteolin, gossypol and gossypolone are much less extensively investigated in the aspect of their anti-inflammatory effects. By administering gossypol as one potential antioxidant inhibitor orally and intrarectally, Fitzpatrick et al discovered the anti-inflammatory activity of gossypol in an *in vivo* model of colitis around 30 years ago (Fitzpatrick et al. 1990). Regarding the mechanism underlying the anti-inflammatory effect, both inhibition of lymphocyte proliferation and decreasing expression of inflammatory cytokines have been suggested. Both *in vivo* and *in vitro*, gossypol is able to significantly inhibit the proliferation of mouse lymphocytes in a dose-dependent manner (Xu et al. 2009). Similar to luteolin, gossypol has been reported to show a suppressive effect on the production of LPS-induced inflammatory mediators such as TNF-α, IL-6 and IL-1β both in vitro and in a mouse model of lung injury in vivo (Huo et al. 2013). In the current study, I showed that both gossypol and gossypolone are able to inhibit IC-stimulated neutrophil adhesion and ROS production, providing additional mechanisms underlying their anti-inflammatory activity.

In the *ex vivo* model for EBA used in this study, gossypolone significantly and dramatically inhibited the IC-induced neutrophil tissue damage. Although not significant, such effect was also observed for gossypol. These results suggest that gossypol and its derivatives could be potential therapeutics for EBA. However, the safety and efficacy of these compounds need to be further evaluated in animal studies *in vivo*.

In conclusion, I screened a natural product library containing 800 natural compounds in order to identify pharmacologically relevant molecules capable in inhibiting IC-induced neutrophil adhesion. Eleven compounds identified from the primary screening were further evaluated for their dose-response

relationship and toxic effect. Finally, three natural compounds were tested in an *ex vivo* model for EBA, and luteolin and gossypolone showed dramatic inhibitory effect on anti-COL7 IgG mediated tissue damage. Therefore, this study has identified that luteolin peracetate and gossypolone are able to inhibit the anit-COL7 IgG mediated tissue damage, providing novel potential therapeutics for EBA.

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Declaration

I declare that this thesis has been written without the help of others, no other than the stated auxiliaries have been used, no other application for admission has been placed, and that the thesis has not been presented anywhere else.

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