

Identification of small-molecule elastase inhibitors as antagonists of IL-36 cytokine activation

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Keywords

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IL-1 family cytokines act as apical initiators of inflammation in many settings and can promote the production of a battery of inflammatory cytokines, chemokines and other inflammatory mediators in diverse cell types. IL-36 α , IL-36 β and IL-36 γ , which belong to the extended IL-1 family, have been implicated as key initiators of skin inflammation in psoriasis. IL-36 γ is highly upregulated in lesional skin from psoriatic individuals, and heritable mutations in the natural IL-36 receptor antagonist result in a severe form of psoriasis. IL-36 family cytokines are initially expressed as inactive precursors that require proteolytic processing for activation. The neutrophil granule-derived protease elastase proteolytically processes and activates IL-36 α and IL-36 γ , increasing their biological activity ~ 500-fold, and also robustly activates IL-1 α and IL-33 through limited proteolytic processing. Consequently, inhibitors of elastase activity may have potential as anti-inflammatory agents through antagonizing the activation of multiple IL-1 family cytokines. Using *in silico* screening approaches, we have identified small-molecule inhibitors of elastase that can antagonize activation of IL-36 γ by the latter protease. The compounds reported herein may have utility as lead compounds for the development of inhibitors of elastase-mediated activation of IL-36 and other IL-1 family cytokines in inflammatory conditions, such as psoriasis.

IL-1 family cytokines, which include the recently described IL-36 α , IL-36 β and IL-36 γ proteins, are among the first cytokines produced in response to infection or injury and play key roles in the initiation of inflammation as a consequence [1–4]. IL-1 family cytokines can initiate the production of many additional cytokines from diverse cell types, such as tissue macrophages and dendritic cells, as well as

keratinocytes and endothelial cells lining local blood vessels [5–11]. IL-36 α , IL-36 β and IL-36 γ are encoded by distinct genes, but all signal via the same receptor, and much evidence now suggests that one or more of these cytokines play an important role in psoriasis [12–21].

Individuals that carry loss-of-function mutations in the IL-36 receptor antagonist (IL-36RA) display a

Abbreviations

AAPV-AMC, methoxy-succinyl-Ala-Ala-Pro-Val-7-amino-4-trifluoromethylcoumarin; ABS, absorbance; AZD9668, Alvelestat; CatG, cathepsin G; CGi, cathepsin G inhibitor; CTRL, control; CXCL1, chemokine (C-X-C motif) ligand 1; DHPI, dihydropyrimidine; FLF-sBzl, succinyl-Phe-Leu-Phe-thiobenzyl ester; H&E, haematoxylin and eosin; HNE, human neutrophil elastase; IL, interleukin; IL-36RA, interleukin-36 receptor antagonist; IL-36R, interleukin-36 receptor; NE, neutrophil elastase; NEi, neutrophil elastase inhibitor; PMA, phorbol 12-myristate 13-acetate; PR3, proteinase-3; PRB, protease reaction buffer; Rec, recombinant; RFU, relative fluorescence unit; RPMI, Roswell Park Memorial Institute; SCID, severe combined immune-deficient; SN, supernatant; TNF, tumour necrosis factor; UT, untreated; VS, virtual screening; WEHD-AMC, Ac-Trp-Glu-His-Asp-7-amino-4-trifluoromethylcoumarin; z-VAD-fmk, carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone.

severe form of psoriasis, called generalized pustular psoriasis [16–20]. Furthermore, expression of all three IL-36 family members has been found to be dramatically elevated (100-fold) in skin biopsies from individuals with the most common form of psoriasis, psoriasis vulgaris, compared with nonlesional skin from the same individuals, or nonaffected controls [13,14]. This suggests that deregulated IL-36 cytokine signalling is sufficient to initiate psoriatic-type skin inflammation. Moreover, deregulated expression of IL-36 α in the mouse leads to a psoriasis-like condition that is exacerbated with the skin irritant, phorbol acetate [12]. In addition, transplantation of human psoriatic lesions onto immunodeficient (SCID) mice produces a psoriasis-like condition that is ameliorated through IL-36 receptor neutralization [13].

As is common within the extended IL-1 cytokine family, IL-36 α , IL-36 β and IL-36 γ are all expressed as leaderless cytokines that lack biological activity unless cleaved within their N termini [11,22,23]. Thus, limited proteolytic processing of IL-36 family cytokines to remove up to 15 N-terminal amino acids dramatically increases their pro-inflammatory activity by over 500-fold [11,22,24,25]. We have recently reported that the neutrophil-derived proteases cathepsin G (CatG) and elastase are potent IL-36-processing proteases, with elastase playing a major role in the processing and maturation of IL-36 α and IL-36 γ [11,25]. Furthermore, IL-1 α and IL-33 are also proteolytically processed and activated by elastase [reviewed in Ref. 26]. Because deregulated IL-36 cytokine activation appears to be instrumental in the initiation of inflammation in the skin barrier, inhibitors of IL-36 activation and/or downstream activity are likely to have potential for the treatment of inflammatory skin conditions. Neutrophil infiltrates are common in psoriasis, and these cells are the major source of elastase [27–29]. Thus, small-molecule inhibitors of neutrophil elastase (NE) may have significant potential as inhibitors of IL-36 activation in psoriasis.

Here, we have used *in silico* screening approaches to identify small-molecule inhibitors of elastase, followed by functional testing of candidates. We show that these inhibitors are capable of antagonizing elastase-mediated processing and activation of IL-36 γ , suggesting that these compounds may be useful leads for the generation of therapeutic modulators of IL-36 cytokine activity in inflammatory conditions.

Results

Elastase processes and activates IL-36 γ

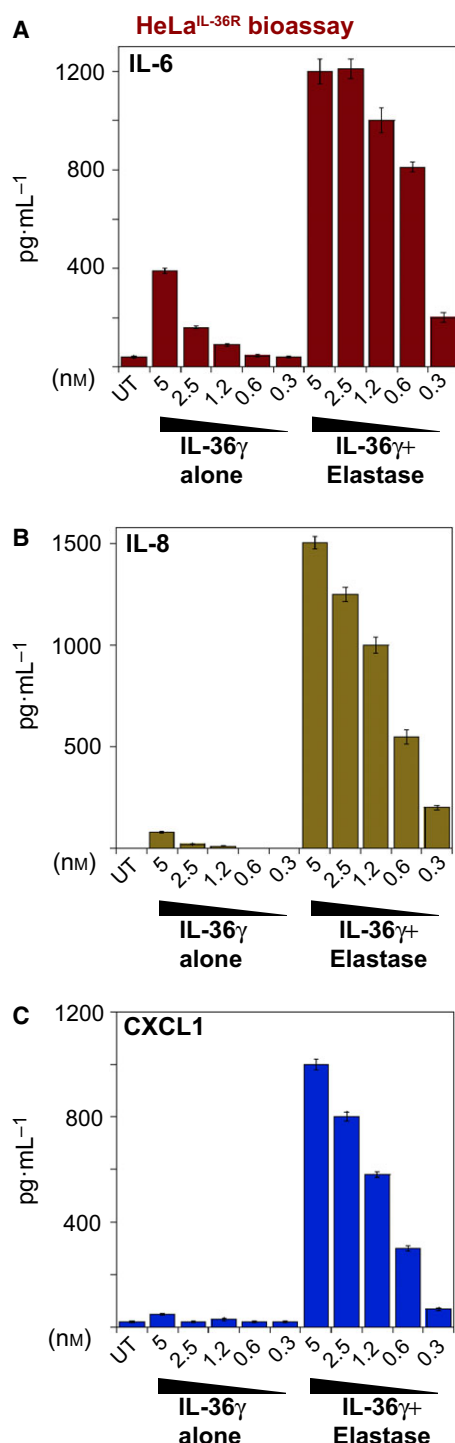
Similar to other members of the extended IL-1 family, such as IL-1 β and IL-18 [26], IL-36 cytokines

possess minimal pro-inflammatory activity as full-length proteins and require N-terminal processing for activation [11,22]. However, as we have recently reported [11,25], IL-36 γ is robustly activated upon incubation with elastase (Fig. 1), a protease that is released in large quantities into the extracellular space from the cytoplasmic granules of activated neutrophils. Elastase processes IL-36 γ at Val15, liberating a new N terminus that unleashes the pro-inflammatory activity of this cytokine, possibly through provoking a conformational change in the latter or through eliminating steric interference within the receptor-binding domain of this cytokine [11]. Of note, previous studies have shown that IL-36 γ is dramatically upregulated at the mRNA and protein levels in lesional skin from psoriasis patients, compared with unaffected skin from the same individuals, or from control subjects [13,14,24,30]. Coupled with observations that loss-of-function mutations in the natural IL-36 receptor antagonist promote a highly severe form of psoriasis [16–20], this suggests that IL-36 γ may be an important driver of the inflammation seen in this condition.

Migration of circulating neutrophils into peripheral tissues is a major amplifier of inflammation and is commonly seen in psoriatic lesions. Neutrophil-derived proteases such as elastase and CatG, although generally thought of as antimicrobial enzymes, are also potent instigators of inflammation [4,11,25,26], most likely through processing and activation of IL-1 family cytokines such as IL-36 α and IL-36 γ . Therefore, inhibitors of NE may have therapeutic potential as anti-inflammatory agents through antagonizing processing and activation of multiple IL-1 family cytokines.

Identification of candidate elastase inhibitors using an *in silico* screening approach

To identify novel small-molecule inhibitors of elastase, we performed *in silico* screening of the elastase crystal structure with an in-house compound library comprised of over 100 000 unique molecular entities (Saint Petersburg Technical University). Using molecular dynamics simulation, compounds were docked in multiple poses into the substrate-binding pocket of elastase, as illustrated by the examples presented in Fig. 2. Using this approach, and guided by known inhibitors of elastase such as dihydropyrimidine (DHPI) (Fig. 2A,B) and Alvelestat/AZD9668 (Fig. 2C,D), we identified a small molecule, designated LCB016, which fit the binding parameters expected of a candidate inhibitor (Fig. 2E,F).



LCB016 and derivatives thereof exhibit elastase-inhibitory activity

LCB016 has an azolactone structure (Fig. 3A); therefore, a series of azolactone analogues were synthesized (LCB001–LCB165; Table 1), which were then assessed

Fig. 1. IL-36 γ is processed and activated by NE. HeLa^{IL-36R} cells were either left untreated or were treated with the indicated concentrations of full-length recombinant human IL-36 γ (ranging from 5 to 0.3 nM), or the same amounts of IL-36 γ that had been pre-incubated for 2 h at 37 °C with purified HNE (50 nM). Twenty-four hours after incubation with either full-length or elastase-processed IL-36 γ preparations, cytokine concentrations in the culture SNs were determined by ELISA. The following cytokines were measured: (A) IL-6, (B) IL-8 and (C) CXCL1. Results shown are representative of at least three independent experiments. Error bars represent the mean $\pm$ SEM of triplicate determinations from a representative experiment.

for their ability to antagonize elastase activity, initially using a synthetic substrate (AAPV-AMC) hydrolysis assay. As Fig. 3B demonstrates, LCB016 and several of its derivatives were found to inhibit NE activity as assessed by the ability of these compounds to antagonize hydrolysis of the synthetic elastase substrate peptide Suc-AAPV-AMC. Substitution of the bromine in LCB016 by hydrogen (LCB108, LCB109, LCB111) led to a significant decrease in calculated binding energy to elastase (data not shown), and consequently to diminished inhibitor potency (Fig. 3B). Substitution of the ethoxy group to smaller methoxy in LCB016 also led to a decrease in binding energy (Table 1 and Fig. 3B). Furthermore, the presence of the benzyloxy group was found to be essential to the binding (data not shown).

Based on these preliminary screening results, we performed titrations of LCB016 and several of its derivatives (LCB091, LCB092, LCB095, LCB113, LCB161, LCB163 and LCB164) against purified elastase (Fig. 4). Once again, we observed reproducible elastase-inhibitory activity of LCB016 and several of its derivatives, with LCB092 and LCB113 exhibiting greater potency than LCB016 (Fig. 4). We also performed time-course analyses of LCB016 and the most promising derivatives over a range of drug concentrations, and as Fig. 5A illustrates, LCB092, LCB113 and LCB116 exhibited the greatest potency towards elastase.

Specificity of candidate elastase inhibitors towards other inflammatory proteases

There is approximately 56% structural similarity between NE and proteinase-3 (PR3), and these proteases exhibit almost identical substrate preferences [11]. Thus, LCB016 and its derivatives also exhibited some inhibitory activity towards PR3, as expected (Fig. 5B). However, no cross-inhibitory effect was observed towards the other major neutrophil protease, CatG (Fig. 6A). Furthermore, caspase-1, which plays

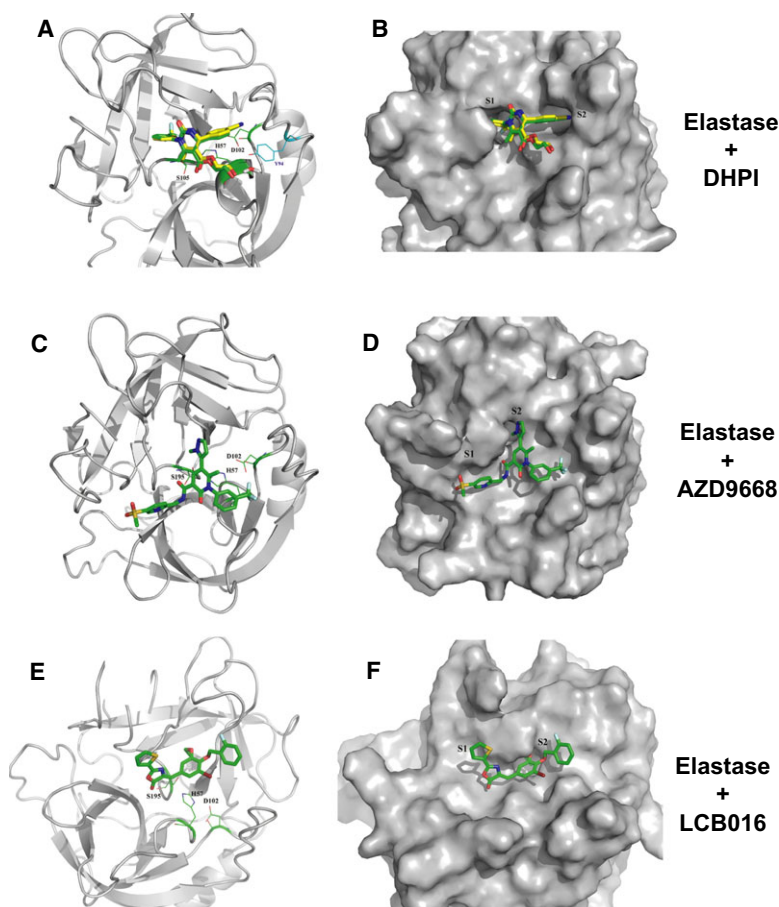


Fig. 2. Structure-based *in silico* screening strategy to identify candidate elastase inhibitors. (A, B) *In silico* docking of the elastase crystal structure with the elastase inhibitor DHPI. (C, D) *In silico* docking of the elastase crystal structure with the elastase inhibitor Alvelestat/AZD9668. (E, F) Representation of the binding pose of newly identified LCB016 inhibitor and interaction interface of LCB016 in the active site of NE. Magenta arrow represents H-bond, and green represents π - π contacts.

a key inflammatory role through processing and activation of IL-1 β and IL-18, was also unaffected by the candidate elastase inhibitors (Fig. 6B). Collectively, these data suggest that the compounds identified herein exhibit good specificity towards NE.

Small-molecule elastase inhibitors can antagonize IL-36 γ activation and cytokine production downstream

Based on the synthetic substrate hydrolysis data, six compounds (LCB016, LCB090, LCB092, LCB113, LCB115 and LCB116) were selected for further testing in the more physiologically relevant context of elastase-mediated IL-36 γ processing. Here, we incubated recombinant full-length IL-36 γ in the presence or absence of elastase, either alone or in combination with the candidate elastase inhibitors (Fig. 7A). The products of the latter reactions were then incubated with HeLa^{IL-36R} cells, which respond to active (i.e. elastase-processed) IL-36 γ by secreting a range of pro-inflammatory cytokines and chemokines such as IL-6,

IL-8 and CXCL1 (Fig. 7A) [11,23]. As Fig. 7B illustrates, while LCB016 and some of its derivatives clearly suppressed elastase-mediated activation of IL-36 γ , as indicated by the inhibition of IL-6 synthesis from HeLa^{IL-36R} cells, LCB092 and LCB113 exhibited greater potency in this regard, in agreement with the synthetic substrate hydrolysis assays (Fig. 5A).

To explore whether the inhibitors identified herein could also inhibit IL-36 γ processing and activation by elastase released from activated neutrophils, we activated purified human neutrophils via treatment with phorbol 12-myristate 13-acetate (PMA; Fig. 8A). The latter treatment triggered the release of multiple neutrophil granule proteases, including elastase, PR3 and CatG, into the extracellular space as expected (Fig. 8B). Using activated neutrophil supernatants (SNs) as a source of elastase to process and activate IL-36 γ , we observed that the lead compounds (LCB016, LCB092 and LCB113) robustly suppressed activation of the latter by neutrophil-derived proteases (Fig. 8C). As we have reported previously [11], IL-36 β is predominantly activated by CatG, and in

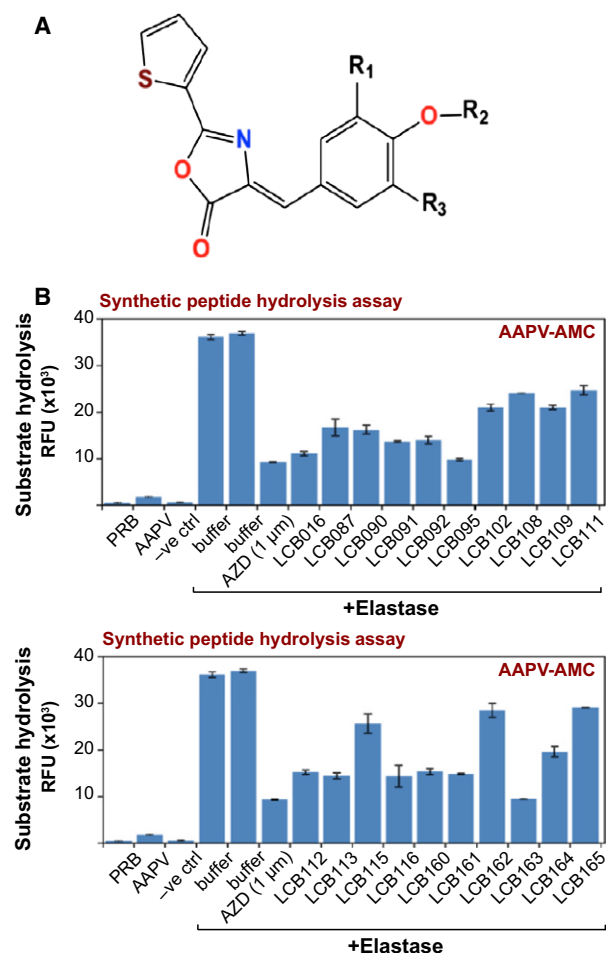


Fig. 3. Several candidates identified by *in silico* screening exhibit inhibitory activity towards purified elastase. (A) General structural formula of LCB016 NE azolactone inhibitor. A series of azolactone analogues were synthesized with substitutions at position R₁, R₂ or R₃. (B) LCB016 and its derivatives (10 μM) were incubated with NE for 2 h at 37 °C. Elastase enzymatic activity was measured by hydrolysis of the elastase substrate peptide Suc-AAPV-AMC (50 μM). AZD9668 (1 μM) was used as a positive control for elastase inhibition.

accordance with this, we found that our elastase-inhibitory compounds had minimal effects on IL-36β activation by neutrophil degranulate preparations. However, inhibition of CatG activity, using a commercial inhibitor of the latter, robustly suppressed activation of IL-36β by activated neutrophil degranulate preparations (Fig. 8D).

In summary, we have identified a number of compounds that are capable of acting as inhibitors of elastase and may have utility as lead compounds designed to suppress inflammation through blocking the actions of this protease against cytokines such as IL-36γ.

Table 1. List of azolactone NE inhibitors and derivatives.

ID number	Substituents
LCB016	R ₁ = Br, R ₂ = 2-fluoro-Bn, R ₃ = OEt
LCB087	R ₁ = Br, R ₂ = 2-chloro-Bn, R ₃ = OMe
LCB090	R ₁ = Cl, R ₂ = 2-chloro-Bn, R ₃ = OMe
LCB091	R ₁ = Br, R ₂ = 2-bromo-Bn, R ₃ = OMe
LCB092	R ₁ = Cl, R ₂ = 2-fluoro-Bn, R ₃ = OEt
LCB095	R ₁ = I, R ₂ = 4-iodo-Bn, R ₃ = OEt
LCB102	R ₁ = Br, R ₂ = Br, R ₃ = Br
LCB108	R ₁ = H, R ₂ = Et, R ₃ = H
LCB109	R ₁ = H, R ₂ = Bn, R ₃ = H
LCB111	R ₁ = H, R ₂ = 2-fluoro-Bn, R ₃ = OEt
LCB112	R ₁ = Br, R ₂ = 4-fluoro-Bn, R ₃ = OEt
LCB113	R ₁ = Br, R ₂ = 2-fluoro-Bn, R ₃ = OMe
LCB115	R ₁ = Br, R ₂ = 3-fluoro-Bn, R ₃ = OEt
LCB116	R ₁ = Br, R ₂ = 2-fluoro-Bn, R ₃ = OEt
LCB160	R ₁ = Cl, R ₂ = 2-fluoro-Bn, R ₃ = OEt
LCB161	R ₁ = I, R ₂ = 2-fluoro-Bn, R ₃ = OEt
LCB162	R ₁ = NO ₂ , R ₂ = 2-fluoro-Bn, R ₃ = OEt
LCB163	Thiophene is substituted on bioisosteric furan ring
LCB164	Thiophene is substituted on benzene ring
LCB165	Azolactone ring is substituted with sulfur

Discussion

Here, we report the identification of several small molecules that exhibit significant inhibitory activity against NE and can antagonize the processing and activation of IL-36γ by the latter. Because elastase has also been implicated in the processing and activation of IL-1α, IL-1β, IL-33 and other cytokines [26], the compounds reported herein are also likely to suppress the activation of multiple members of the extended IL-1 family. Further experiments will explore the latter possibility and will also explore the activity of these compounds in animal models of psoriasis and other inflammatory diseases.

Neutrophil infiltration is a hallmark of a number of skin-related inflammatory diseases. In particular, psoriatic plaques are heavily infiltrated with neutrophils, dendritic cells, macrophages and T cells [27,28,31]. Neutrophils are well-known first responder cells of the innate immune system and play a central role in the early stages of infection or tissue damage [32–34]. Although it is widely appreciated that release of neutrophil proteases (through degranulation or neutrophil extracellular trap [NET] formation) can exert profound antimicrobial effects during infection, the latter proteases can also cause extensive tissue damage and exacerbate inflammation [35–37]. Psoriasis is often preceded by tissue damage or abrasion, called the Koebner reaction [38], which is most likely due to

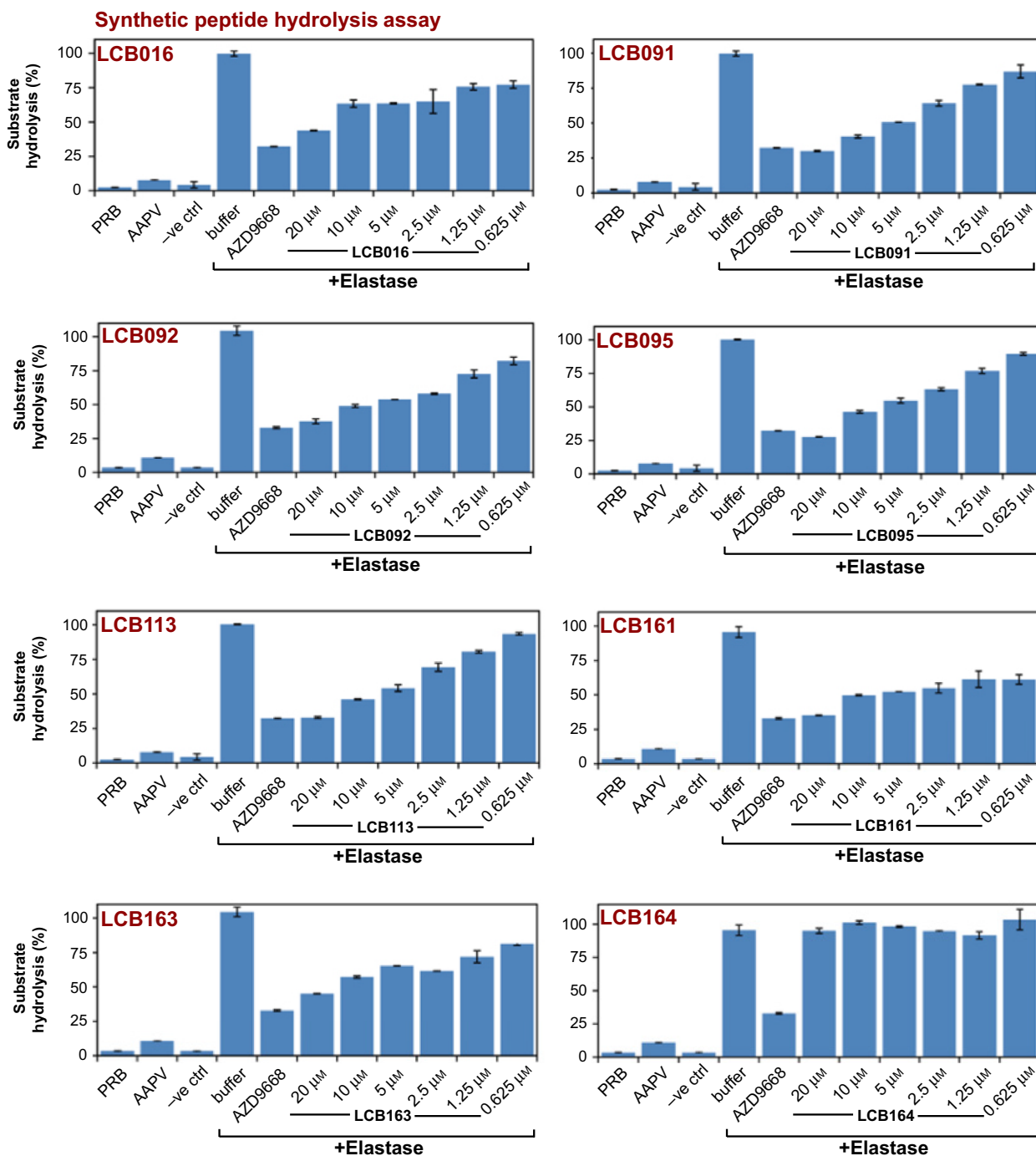


Fig. 4. Azolactone derivatives inhibit elastase activity. LCB016 and its derivatives were titrated against purified NE. Azolactone derivatives were incubated with elastase for 2 h at 37 °C, as indicated. AZD9668 (1 μ M) was used as a positive control for elastase inhibition. Elastase enzymatic activity was measured by hydrolysis of the elastase substrate peptide Suc-AAPV-AMC (50 μ M).

liberation of cytokines such as IL-36 and other IL-1 family cytokines into the extracellular space due to necrosis. Upon release into the interstitial fluid, the latter cytokines can then encounter and become

processed by proteases, such as elastase, liberated from activated neutrophils. Thus, damage to keratinocytes resulting in the liberation of IL-36 cytokines, either as a result of microbial infection or as a

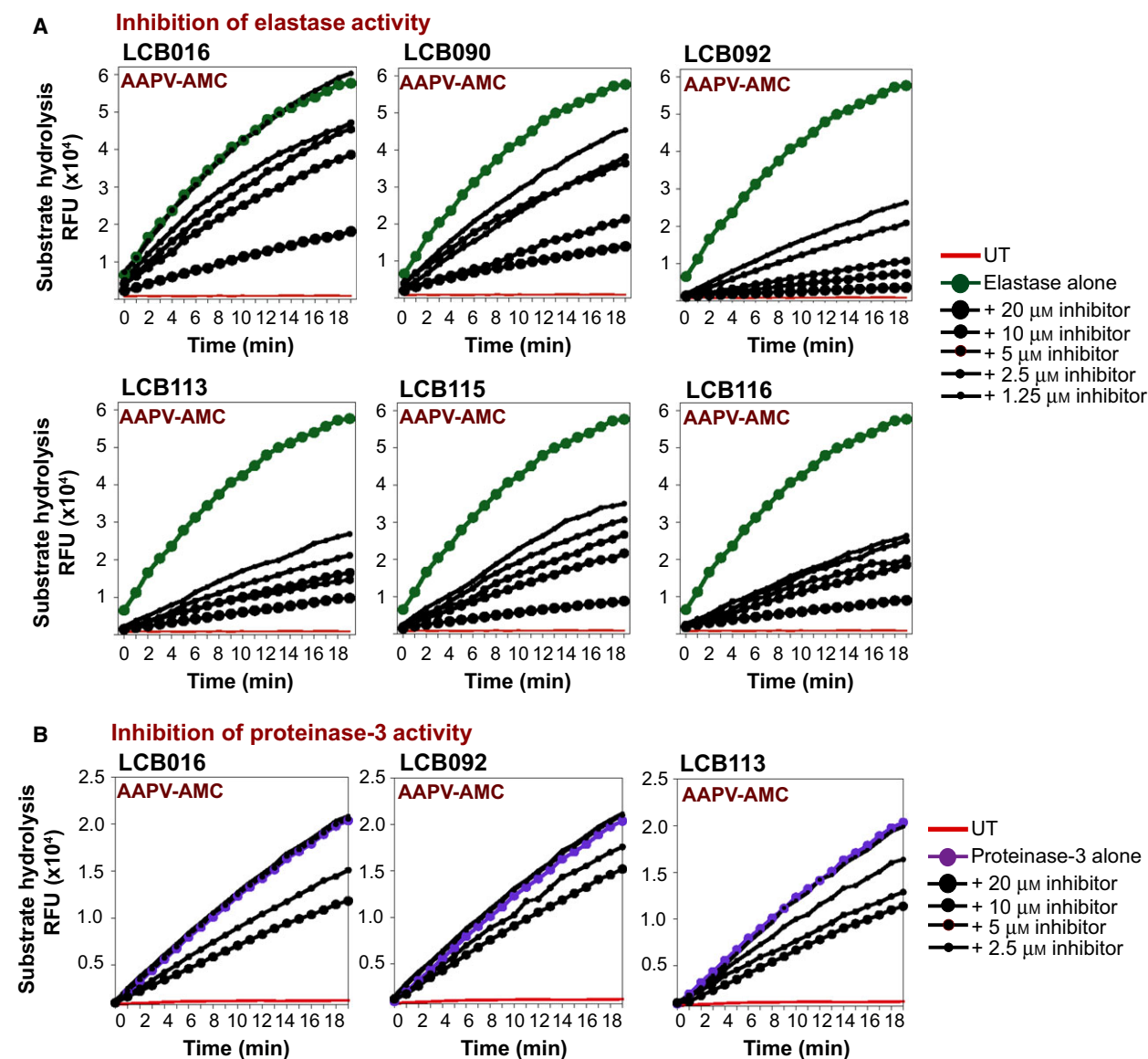


Fig. 5. Kinetic analysis of elastase inhibition and specificity of azolactone derivatives. (A) Hydrolysis of the synthetic peptide substrate AAPV-AMC by a fixed concentration of purified elastase (50 nM), in the presence or absence of the indicated concentrations of the candidate elastase inhibitors. (B) Hydrolysis of the synthetic peptide substrate AAPV-AMC by a fixed concentration of purified PR3 (200 nM), in the presence or absence of the indicated concentrations of the candidate elastase inhibitors.

consequence of tissue trauma, may play an important initiating role in psoriasis, especially in individuals lacking endogenous buffers of IL-36 activity, such as deficiency in the IL-36R antagonist [16–20]. Because previous studies have also implicated NE in the activation of IL-1 α [39], IL-1 β [40], IL-18 [41] and IL-33 [42] and IL-36 receptor antagonist [43], our observations also suggest that this protease may serve as a therapeutic target for suppressing the activation of multiple IL-1 family cytokines in inflammatory diseases.

Psoriasis is associated with a pro-inflammatory signature with elevated levels of tumour necrosis factor (TNF), IL-17C and IL-36 among other cytokines. At present, multiple biotherapeutics, directed against cytokines such as TNF, IL-17, IL-12/IL-23 and IL-36, have been approved or are in development for the treatment of psoriasis, as well as other inflammatory conditions. However, although these cytokine-neutralizing approaches are highly effective, they also suffer from several drawbacks including high cost, a necessity for systemic delivery (which can increase vulnerability to

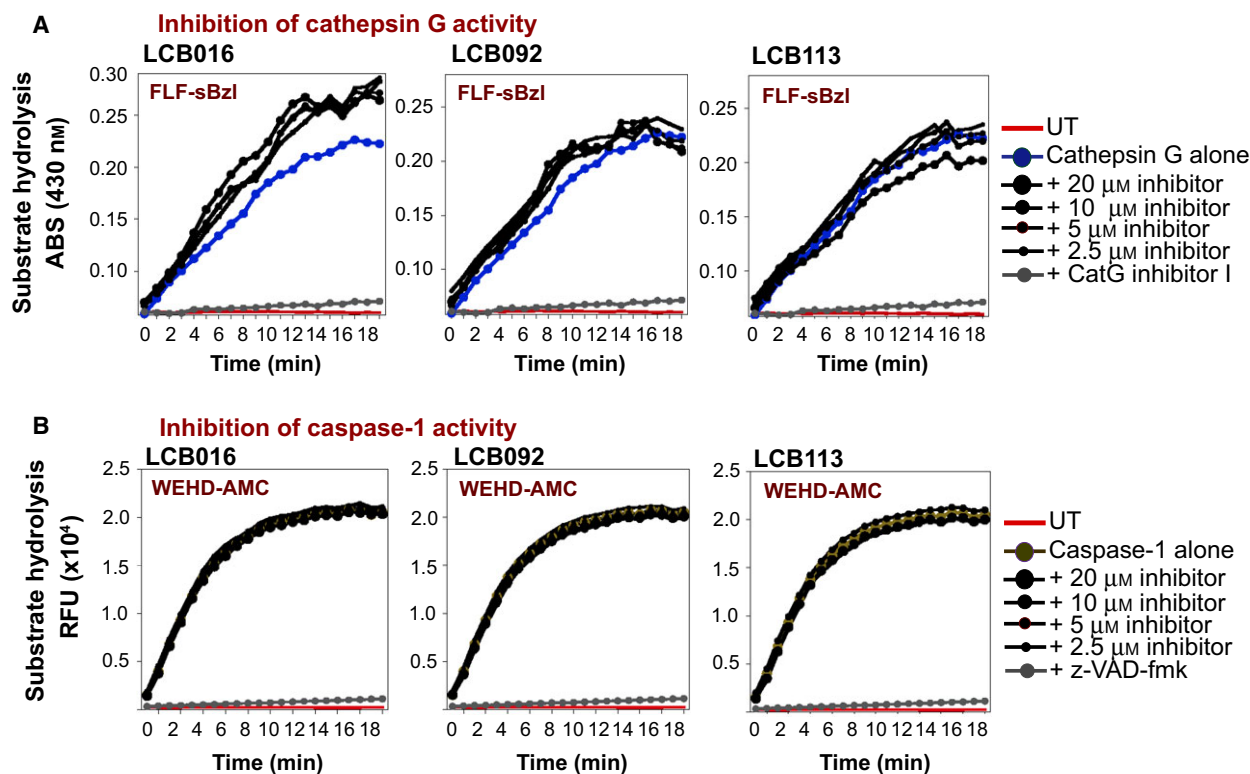


Fig. 6. Azolactone derivatives do not inhibit neutrophil CatG or caspase-1. (A) Hydrolysis of the synthetic CatG substrate FLF-sBzl by a fixed concentration of purified CatG (50 nM), in the presence or absence of the indicated concentrations of the candidate elastase inhibitors. Cathepsin G inhibitor I (CatG inhibitor I, 5 μM) served as a positive control. (B) Hydrolysis of the synthetic caspase-1 substrate WEHD-AMC by a fixed concentration of recombinant human caspase-1 (1 : 100), in the presence or absence of the indicated concentrations of the candidate elastase inhibitors. z-VAD-fmk (5 μM) served as a positive control.

opportunistic infections) and their single-molecule specificity. The need for systemic delivery of cytokine-neutralizing biotherapeutics is particularly problematic due to the neutralization of cytokine throughout the body and not just where it is overproduced. A more desirable approach would be to neutralize cytokine only in the affected lesions. Thus, small molecules, such as the compounds reported herein, have the advantage that they are less costly to produce, can be applied directly to affected areas of skin and may simultaneously suppress activation of multiple cytokines that are processed and activated by elastase [11,29].

Experimental procedures

Materials

Suc(OMe)-AAPV-AMC was purchased from PeptaNova (Sandhausen, Germany); elastase inhibitor Alvelestat/AZD9668 was purchased from MedChem Express (Solentuna, Sweden). Purified neutrophil-derived elastase was

purchased from Serva (Heidelberg, Germany). Purified neutrophil PR3 and neutrophil CatG were purchased from Calbiochem (Merck), Cork, Ireland. Suc-FLF-SBzl, Ac-WEHD-AMC and z-VAD-fmk were purchased from Bachem (Bubendorf, Switzerland). Chemical inhibitors of CatG (cathepsin G inhibitor I) and of elastase (elastase inhibitor IV) were purchased from Calbiochem. Unless otherwise indicated, all other reagents were purchased from Sigma Aldrich (Ireland) (Wicklow, Ireland).

Cell culture

HeLa cells were cultured in RPMI media (Gibco (BioSciences), Dublin, Ireland), supplemented with 5% FBS. HeLa.IL-36R cell lines were generated by transfection with pCXN2.empty or pCXN2.IL-1Rrp2 (IL-36R) plasmids followed by selection using G-418 antibiotic (Sigma). IL-36R-overexpressing clones were expanded from a single cell. Clones were selected by demonstration of acquired optimal responsiveness to active forms of IL-36 via ELISA. All cells were cultured at 37 °C in a humidified atmosphere with 5% CO₂.

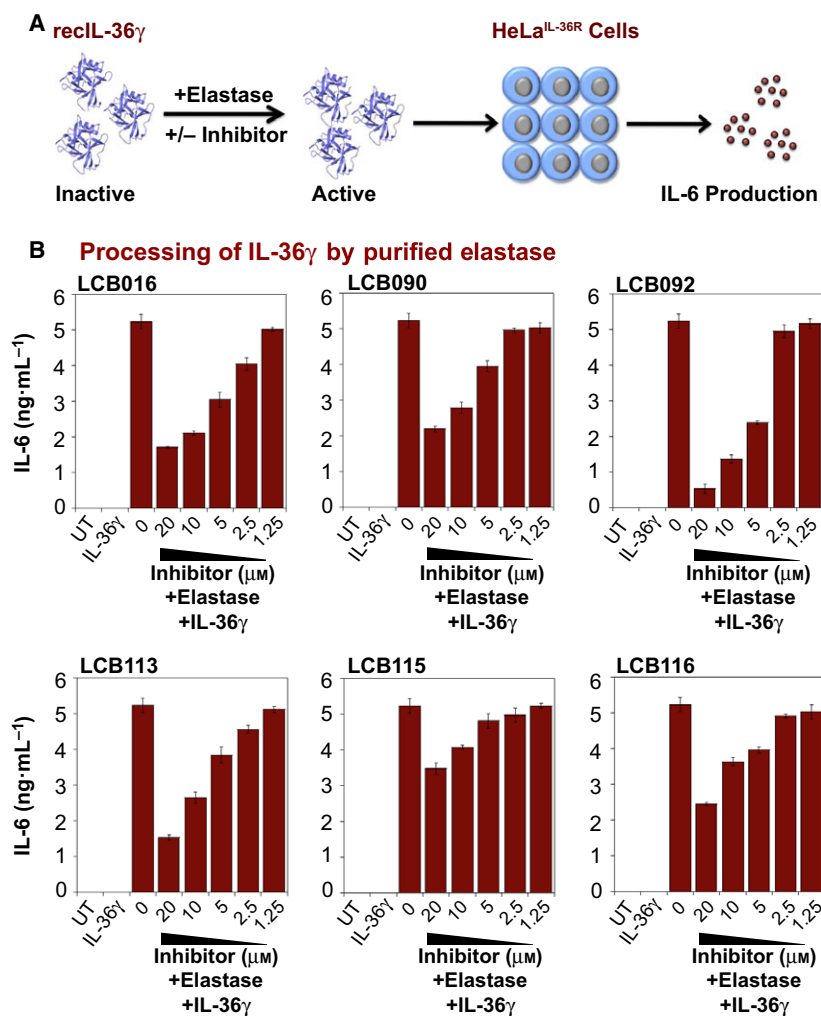


Fig. 7. Inhibition of elastase-mediated IL-36 γ activation by LCB016 and derivatives. (A) Schematic overview of the IL-36 activity bioassay. (B) Purified recombinant IL-36 γ was incubated for 2 h at 37 °C in the presence or absence of elastase (50 nM), either alone or in combination with the indicated small-molecule elastase inhibitors. Reaction products were then added to HeLa^{IL-36R} cells, such that the final concentration of IL-36 γ was 500 pM. After 24 h, IL-6 cytokine concentrations in cell culture SNs were determined by ELISA. Results shown are representative of at least three independent experiments. Error bars represent the mean $\pm$ SEM of triplicate determinations from a representative experiment.

Expression and purification of recombinant proteins

Full-length IL-36 β and IL-36 γ were generated by cloning the human coding sequence in frame with the polyhistidine tag sequence in the bacterial expression vector pET45b. Protein was expressed by addition of 600 μ M IPTG to exponentially growing cultures of BL21 strain *Escherichia coli* followed by incubation for 3 h at 37 °C. Bacteria were lysed by sonication and polyhistidine-tagged proteins were captured using nickel/NTA agarose (Qiagen (UK), Manchester, UK), followed by elution into PBS, pH 7.2, in the presence of 100 mM imidazole. Recombinant polyhistidine-tagged caspase-1 and caspase-3 were expressed and purified as described previously [44].

In silico screening and molecular modelling

Ligand 3D structures were prepared with LigPrep suite in OPLS_2005 force field. The Schrodinger package was used

for molecular modelling studies. Glide was used for SM virtual screening (VS) and docking studies; the ligands from the in-house SM library were treated as flexible. The standard precision protocol was used for VS. The positions of the identified azolactone hit compounds were specified within extra-precision protocol. Azolactone derivatives were redocked as Z-isomers corresponding XRD data. The X-ray human neutrophil elastase (HNE) structure (PDB ID 1HNE) was used for *in silico* screening; the water and cocrystallized molecules were removed from the protein model. Hydrogens were added using PROPKA program pH 7.4 and their positions optimized. The 25 $\times$ 25 $\times$ 25 Å³ box (with 12 Å diameter ligand mid-point), centred on the coordinates of ⁵⁷His residue (active site), was used for grid map calculations; the ⁹⁴Tyr, ⁵⁴Ser, ¹⁹⁵Ser and ²¹⁴Ser hydroxyl groups were allowed to rotate during docking runs. For the validation of the docking protocol, the DHPI ligand was blindly docked to the HNE structure (PDB ID 3Q77) and the poses of docked ligand and cocrystallized with HNE were aligned (Fig. 2) showing good theory-to-experiment correspondence.

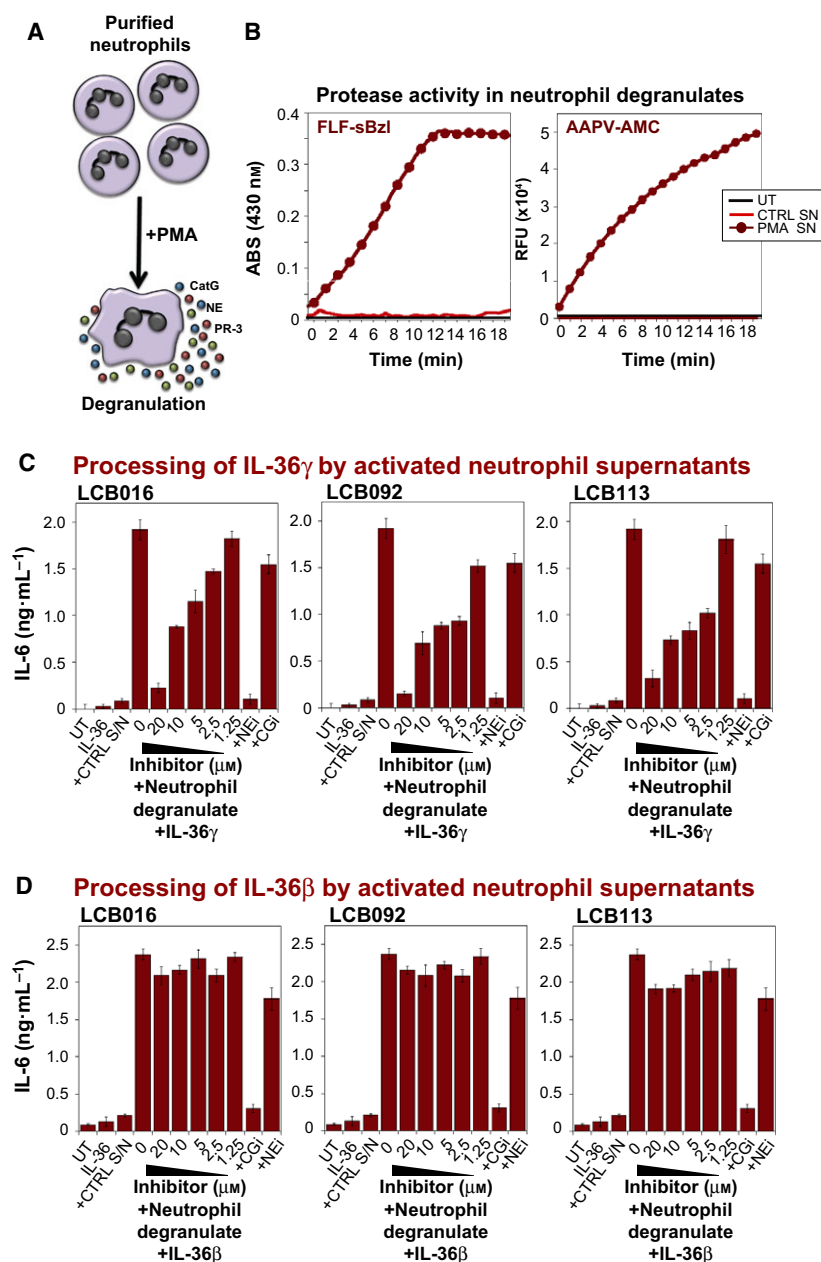


Fig. 8. Novel elastase inhibitors suppress IL-36 γ activation by proteases released from human neutrophil degranulate SNs. (A) Schematic representation of PMA-induced neutrophil degranulation. (B) Assessment of elastase and CatG activity in SNs from untreated versus PMA-activated human neutrophils. Protease activity was assessed through hydrolysis of FLF-sBzl (CatG activity) or AAPV-AMC (elastase/PR3 activity), as before. (C) Purified recombinant human IL-36 γ was incubated for 2 h at 37 °C in the presence or absence of control (unstimulated) or PMA-activated neutrophil SNs (1 : 8), either alone or in combination with the indicated small-molecule elastase inhibitors. Neutrophil cathepsin G inhibitor I (CGI) and elastase inhibitor IV (NEI) served as controls. Reaction products were then added to HeLa^{IL-36R} cells, such that the final concentration of IL-36 γ was 500 pM. After 24 h, IL-6 cytokine concentrations in cell culture SNs were determined by ELISA. (D) Purified recombinant IL-36 β was incubated for 2 h at 37 °C in the presence or absence of control (unstimulated) or PMA-activated neutrophil SNs (1 : 8), either alone or in combination with the indicated small-molecule elastase inhibitors. Neutrophil cathepsin G inhibitor I (CGI) and elastase inhibitor IV (NEI) served as controls. Reaction products were then added to HeLa^{IL-36R} cells, such that the final concentration of IL-36 β was 500 pM. After 24 h, IL-6 cytokine concentrations in cell culture SNs were determined by ELISA. Results shown are representative of at least three independent experiments. Error bars represent the mean $\pm$ SEM of triplicate determinations from a representative experiment.

Protease activity assays

Reactions (50 μ L, final volume) were carried out in protease reaction buffer (PRB) (50 mM HEPES [pH 7.2], 75 mM NaCl and 0.1% 3-[(α -cholamidopropyl)dimethylammonio]-1-propanesulfonate [CHAPS] [2 mM DTT added only for caspases]) containing Ac-WEHD-AMC and Suc(OMe)-AAPV-AMC (50 μ M, final concentration). Samples were measured using an automated fluorimeter (SPARK 10M; TECAN (UK), Reading, UK) at wavelengths of 430 nm (excitation) and 535 nm (emission). For the Suc-FLF-SBzl hydrolysis assay, the substrate was diluted to a final concentration of 300 μ M in PRB (50 mM HEPES [pH 7.2], 75 mM NaCl, 0.1% CHAPS and 300 μ M 5,5-dithiobis(2-nitrobenzoic acid [DTNB]). Cathepsin G hydrolyses the synthetic substrate Suc-FLF-sBzl with the release of the thiobenzyl group. The free thiobenzyl group reacts with DTNB and produces a chromophore (3,3',5,5'-tetramethylbenzidine [TNB]) that absorbs at 430 nm. Samples were measured by automated fluorimeter (SPARK 10M TECAN (UK)).

Protease cleavage assays

Reactions (40–100 μ L, final volume) were carried out in PRB (50 mM HEPES [pH 7.2], 75 mM NaCl and 0.1% CHAPS) for 2 h at 37 °C. For IL-36 bioassays, IL-36 cytokines were typically cleaved at a 50 nM concentration and subsequently diluted onto target cells at a final concentration ranging from 0.25 to 1 nM.

Purification of primary neutrophils and preparation of degranulates

Primary human neutrophils were purified from donor whole blood using the Ficoll-Hypaque gradient method as described previously [11]. The purity of the cell preparations (> 90%) was determined by H&E staining of cytopins. To prepare degranulates, neutrophils (10^7 per treatment) were stimulated in the presence or absence of 50 nM PMA in Hanks' balanced salt solution/0.25% BSA for 1–3 h at 37 °C in a humidified atmosphere with 5% CO₂. SNs were harvested and clarified by centrifugation at 4 °C (10 000 $\times$ g for 5 min). Neutrophil degranulate aliquots were stored at –80 °C. Experiments involving human samples were carried out in accordance with the regulations of the EU and the Irish Department of Health, and all procedures performed were approved by the Trinity College Dublin Research Ethics Committee (Ethical Approval Number 311217).

Measurement of cytokines and chemokines

Cytokines and chemokines were measured from cell culture SNs using specific ELISA kits obtained from R&D Systems (human IL-6, IL-8, CXCL1). All cytokine assays were carried out using triplicate samples from each culture.

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Author contributions

GPS, PBD, SS-T and EB performed experiments and analysed data; PBD performed the *in silico* screening; CMH, DMC and AZ generated reagents; TM and AVG provided advice, supervision and comments on data; and SJM conceived the project, supervised the study and wrote the manuscript.

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