

Immunomodulatory activity of commonly used drugs on Fc-receptor-mediated human natural killer cell activation

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Abstract Natural killer (NK) cells mediate defense against neoplastic as well as infected cells. Yet, how their effector functions are affected by the large variety of pharmacological compounds commonly in use has not been investigated systematically. Here, we screened 1,200 in-use or previously approved drugs for their biological effect on freshly isolated human peripheral blood-derived NK cells. Mimicking antibody-dependent cellular cytotoxicity (ADCC), known to be important in antibody-based immunotherapies against, e.g., human malignancies, the cells were stimulated by Fc-receptor (CD16) engagement. Cellular responses were assessed by

flow cytometry. Fifty-six compounds that significantly inhibited and twelve that enhanced one or more of the readouts of adhesion, exocytosis, and chemokine production were identified and confirmed as hits. Among the confirmed inhibitors, 80 % could be assigned to one of seven major pharmacological classes. These classes were β 2-adrenergic agonists, prostaglandins, phosphodiesterase-4 inhibitors, Ca^{2+} -channel blockers, histamine H1-receptor antagonists, serotonin/dopamine receptor antagonists, and topoisomerase inhibitors that displayed distinct inhibitory patterns on NK cell responses. Among observed enhancers, interestingly, two ergosterol synthesis inhibitors were identified that specifically promoted exocytosis. In summary, these results provide a comprehensive knowledge base of the effect known drugs have on NK cells. More specifically, they provide an overview of drugs that may modulate NK cell-mediated ADCC in the context of clinical immunotherapies.

The authors of this paper report on their NK assays transparently and comprehensively as per field-wide consensus, allowing the community a full understanding and interpretation of presented data as well as a comparison of data between groups. The electronic supplementary materials of this publication include a MIANKA checklist. For more details, see <http://miataproject.org/>.

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Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
DMSO	Dimethylsulfoxide
FBS	Fetal bovine serum
FSC-H	Forward scatter height
ITAM	Immunoreceptor tyrosine-based activation motif
IFN	Interferon
LFA	Leukocyte functional antigen
MIP	Macrophage-inducible protein
NK	Natural killer cells
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PDE	Phosphodiesterase
SSC-H	Side scatter height
TNF	Tumor necrosis factor

Introduction

Natural killer (NK) cells can kill neoplastic or virally infected cells, as well as activated immune cells [1]. Such killing is mediated by targeted release of cytotoxic proteins from specialized lysosomes [2]. NK cells are typically considered innate lymphocytes, as their activation is controlled by integration of signals from a variety of activating and inhibitory germline-encoded receptors, including the low-affinity Fc-receptor CD16 for antibody-mediated cellular cytotoxicity (ADCC) [1]. In addition to their cytotoxic function, NK cells produce chemokines, such as CCL4 [macrophage inducible protein (MIP)-1 β], and cytokines, such as tumor necrosis factor (TNF) and interferon (IFN)- γ , which orchestrate subsequent immune responses. Impaired NK cells function is implicated in several disorders, including susceptibility to viral infections and certain autoimmune conditions [3, 4]. Furthermore, NK cell functions may be harnessed in therapeutic settings, e.g., cellular immunotherapy or ADCC-dependent antibody-mediated therapy of cancer [5, 6]. Thus, it is of therapeutic interest to understand how pharmacological compounds modulate NK cell functions. In addition, further insights into NK cell pathways that can be targeted with small molecules may provide alternative therapeutic applications.

Currently, the Western world pharmacopoeia comprises over 2,700 unique drugs [7], of which the majority are low-molecular-weight compounds [8]. It is clear that a single compound often acts broadly in target space, i.e., polypharmacology is commonly encountered [8, 9]. Even recently developed compounds, designed to specifically target a single protein, display off-target effects [8, 9]. In-use drugs are in general well characterized with regard to their mechanisms of action through biochemical and cellular assays, and their physiological effects have been studied both in animal models and in humans. Nevertheless, how such

large sets of active compounds affect the function of specific primary human cell types, e.g., NK cells, is less clear.

Hence, we set out to evaluate the activity of a large variety of pharmacological compounds with respect to primary NK cell responses. For this purpose, a sensitive and robust assay was required. CD3⁺CD56^{dim} NK cells represent the largest subset of lymphocytes in human peripheral blood, and the engagement of CD16 on NK cells induces vigorous activation of ADCC [10]. Not only is CD16 essential for antibody-mediated immunotherapy, assays evaluating the response of NK cells to engagement of CD16 can also provide broader insights into the modulation of cytotoxic lymphocyte responses in general, as immune-receptor tyrosine-based activation motif (ITAM)-coupled signaling pathways are shared by many activating lymphocyte receptors.

Here, we have systematically analyzed the activity of the Prestwick Chemical Library, which comprises a large and diverse selection of in-use or previously approved drugs (www.prestwickchemical.com) on NK cells using a robust, high-throughput flow cytometric assay. Readouts of primary CD3⁺CD56^{dim} NK cell activation were adhesion, exocytosis, and chemokine production. Sixty-eight inhibitors and enhancers of cytotoxic lymphocyte responses have been identified and characterized further. These data provide a catalog for understanding of pharmacologically desirable effects as well as side effects for these diverse compounds that may impact NK cell-dependent ADCC, e.g., in the context of immunotherapies or more generally therapies involving the function of cytotoxic lymphocytes.

Materials and methods

Cells

Peripheral blood mononuclear cells (PBMC) from healthy blood donors were freshly isolated from buffy coats by density gradient centrifugation (Lymphoprep, Axis-Shield). Per Buffy coat, 300–600 $\times 10^6$ PBMC were isolated. Cells were incubated overnight in complete media (Roswell Park Memorial Institute 1,640 supplemented with 10 % fetal bovine serum (FBS), L-glutamine, penicillin, and streptomycin; all HyClone). This medium was not specifically pretested for this project, but previous comparisons of media for short-term functional NK cells assays have revealed consistent results. The cells were used for screening and follow-up characterization purposes within 20 h of venipuncture. Cell viability was determined with trypan blue in a Bürker chamber and was above 98 %.

All experiments were performed in research laboratory environments. The protocols have not been validated for clinical use. The studies were reviewed and approved by the Regional Ethical Committee of Stockholm Council.

Prestwick Chemical Library and plate configuration

The entire Prestwick Chemical Library (Prestwick Chemical), encompassing 1,200 compounds, was included in the primary screen. Compounds were delivered and stored ($-20\text{ }^{\circ}\text{C}$) in dimethylsulfoxide (DMSO) at a concentration of 10 mM. Assay ready 96-well plates containing $2\text{ }\mu\text{l}$ of each of the compound solutions in separate wells were prepared followed by heat sealing and storage at $-20\text{ }^{\circ}\text{C}$. Wells in columns 2–11 each contained a single active compound. On the day of each screen, the plates were thawed and $2\text{ }\mu\text{l}$ of DMSO only (negative control) or 5 mM solution of the sarcoma (SRC) kinase inhibitor PP2 (Merck Millipore) in DMSO (positive control) was added to wells A12-D12 and E12-H12, respectively. Thereafter, the solutions were diluted to a concentration of 100 μM by the addition of RPMI media using a non-contact dispenser (Flexdrop, PerkinElmer), and $20\text{ }\mu\text{l}$ of each solution was transferred to 96-well V-bottom plates (Nunc) using a CyBiwell (CyBio) liquid handling instrument equipped with a 96-well dispensing head. This procedure resulted in the entire Prestwick Chemical Library being distributed over 15 assay plates.

Antibodies and fluorescent reagents

For stimulation, an anti-CD16 (3G8, BD Bioscience) monoclonal antibody (mAb) was used. For flow cytometry, fluorochrome-conjugated anti-CD3 (clone S4.1, Invitrogen), anti-CD56 (NCAM16.2, BD Bioscience), anti-CD69 (TP1.55.3, Beckman Coulter), anti-CD107a (H4A3, BD Bioscience), anti-IFN- γ (B27, BD Bioscience), anti-MIP-1 β (D21-1351, BD Bioscience), and anti-TNF (Mab11, BD Bioscience) mAbs were used. In addition, biotinylated conformation-specific anti-leukocyte functional antigen (LFA)-1 (mAb24, Abcam) mAb as well as fluorochrome-conjugated streptavidin (BD Bioscience) was used. A fixable cell marker (Invitrogen) was used for assessment of cellular viability. For cellular barcoding, a cell trace violet dye (Invitrogen) was used.

Assay description

For primary screening of the effect of the examined compounds on NK cell responses, $155\text{ }\mu\text{l}$ of cell suspension in complete medium (3×10^6 PBMC/ml) was dispensed using a Multidrop (Thermo Scientific) into wells containing $20\text{ }\mu\text{l}$ of 100 μM compound solutions. The cells were pre-incubated with compounds for 30 min at $37\text{ }^{\circ}\text{C}$. Thereafter, $25\text{ }\mu\text{l}$ of 0.5 $\mu\text{g/ml}$ anti-CD16 in complete medium was added using a FlexDrop, and the mixture incubated for 60 min at $37\text{ }^{\circ}\text{C}$, resulting in a 10- μM compound assay concentration. Following stimulation, the cells were

pelleted (i.e., PBMC suspensions were centrifuged for 3 min at 450g). The supernatant was then discarded, and surface stained with $50\text{ }\mu\text{l}$ phosphate-buffered saline (PBS) supplemented with 2 % FBS (staining solution) and antibodies to CD3, CD56, CD107a, a conformation-specific LFA-1 antibody as well as a fixable dead cell stain (LIVE/DEAD fixable dead cell stain kit, Invitrogen) for 30 min at $4\text{ }^{\circ}\text{C}$. Thereafter, the cells were pelleted, and cells were stained in $50\text{ }\mu\text{l}$ staining solution supplemented with fluorochrome-conjugated streptavidin. After 20 min, the cells were pelleted and the cells fixed with $50\text{ }\mu\text{l}$ 2 % paraformaldehyde in PBS, followed by incubation for 10 min at $4\text{ }^{\circ}\text{C}$. Subsequently, the plates were pelleted, after which the cells were permeabilized, and stained intracellularly with anti-MIP-1 β antibody diluted in $50\text{ }\mu\text{l}$ BD perm/wash buffer (BD Bioscience) for 30 min at $4\text{ }^{\circ}\text{C}$. Finally, the cells were pelleted, and the cells were resuspended in $60\text{ }\mu\text{l}$ staining solution. Data was acquired on a flow cytometer (LSR Fortessa instrument with a high-throughput sampler, BD Bioscience) within 20 h of staining followed by analyses (FlowJo, v9.4, Tree Star) and Excel 2008 software (version 12.3.5, Microsoft).

In the dose–response assay, a few changes were made to the original screening protocol. PBMC were incubated with compound doses from 0.04 to 25 μM and stimulated for 4 h in the presence of GolgiPlug (1:1,000, BD Bioscience). Donor PBMCs were fluorescently barcoded during fixation to multiplex donor responses. In addition, antibodies to IFN- γ , TNF, and CD69 were included in the intracellular staining step.

Analysis of flow cytometric data

Single-stained bead controls (CompBeads, BD Biosciences) were used for setting an electronic compensation matrix. For quantification of NK cell responses, lymphocytes were first gated on side scatter height (SSC-H) versus forward scatter height (FSC-H) contour plots, followed by gating of single cells on FSC-H versus FSC-Area (FSC-A) contour plots, viable cells on a dead cell marker versus SSC-A contour plot, and CD3 $^{-}$ CD56 $^{\text{dim}}$ NK cells on a CD3 versus CD56 contour plot. Finally, the magnitude of responses was quantified as the frequency of responding cells in specific read-out channels (Supplementary figure 1A). For exclusion of toxic compounds, the frequency of all acquired events that stained with a dead cell marker was quantified (Supplementary figure 1B). For exclusion of compounds that caused fluorescent base-line shifts of responses in read-out channels, viable lymphocytes were gated as detailed, followed by gating on CD3 $^{+}$ CD56 $^{-}$ T lymphocytes on a CD3 versus CD56 contour plot. The magnitude of baseline shift was quantified as the frequency of positive cells in read-out channels with a gate set to 5 %

in the DMSO controls (see Supplementary Figure 1C for gating strategy). In the screen, after exclusion of all the compounds meeting any exclusion criteria ($n = 82$), the number of gated NK cells ranged from 295 to 3,677. The number of gated NK cells in the first and 99th percentile as well as the median cell number was 548, 2,959, and 1747s, respectively.

In the dose–response assay, the same gating strategy as above was applied. Furthermore, gates for IFN- γ , TNF, and CD69 were added (the gating strategy is depicted in Supplementary figure 1D). In this experiment, the number of total gated NK cells ranged from 107 to 12,509. The number of cells in the first and 99th percentile as well as the median cell number was on average 822, 1,753, and 3,549 per donor, respectively.

Flow cytometry data will be made available upon request

Normalizations and statistical calculations

The quality of the data on each individual plate was followed by looking at the signal to background values and, more importantly by calculating the Z'-factor based on the controls, which were available in all plates in column 12 [11]. The Z'-factor is a widely used statistical measurement of the signal-to-noise ratio and takes into account variabilities in both positive and negative control samples. A Z'-factor value of more than 0.5 is considered a good parameter for pharmacological screening, and values exceeding 0.7 are considered excellent [11].

Because average responses in the controls varied somewhat from plate to plate, data were normalized on a per plate basis. Normalization of values representing the inhibition of functional readouts was calculated as $Inhibition_y = 100 \times (1 - (x_y - x_{PP2z}) / (x_{DMSOz} - x_{PP2z}))$, where x_y denominates the percentage of responding CD3⁺CD56^{dim} cells in well y of plate z , whereas x_{DMSOz} and x_{PP2z} denote the average percentage of responding CD3⁺CD56^{dim} cells in DMSO and PP2 control wells of plate z , respectively. Normalization of toxicity results was calculated as $Toxicity_y = 100 \times (x_y / x_{DMSOz})$, where x_y denominates the percentage of dead cell marker-positive events in well y of plate z , and x_{DMSOz} denominates the average percentage of dead cell marker-positive events in DMSO control wells of plate z . The same formula was used for normalization of base-line shifts.

For identification of hits as well as exclusion of toxic and fluorescent compounds, a quartile-based selection method was used [12]. The quartile-based selection method was calculated as $Q_1 - 2c(Q_2 - Q_1)$ for negative outliers and $Q_3 + 2c(Q_3 - Q_2)$ for positive outliers, where c determined by the desired α -error rate, and Q are the respective quartiles calculated on cumulative data points for all plates

with the exception of wells containing the PP2 control compound. Only 0.27 % of α -errors were accepted, corresponding to the use of three standard deviations as a threshold for hit determination [13].

Clustering based on molecular fingerprints, pharmacological properties, and functional effects on NK cells

The Prestwick collection of approved drugs was structurally stratified using modules in the cheminformatics tool Canvas (version 1.5, Schrödinger). Binary fingerprints, molprint2D from 2-dimensional structures, were used [14, 15]. The compounds were clustered by similarity using hierarchical clustering based on Tanimoto coefficient metrics. Setting the merging distances to 0.93 resulted in 88 clusters containing 2–166 compounds and 21 structurally distinct compounds. The merging distance setting was established by manual inspection of the clusters to ensure that closely related compounds were contained within the same cluster.

Data regarding the pharmacological properties of hit compounds were retrieved from the database PubChem Substance (<http://www.ncbi.nlm.nih.gov/pcsubstance> between March 10 and 25, 2013). For eight compounds, no pharmacological information was available on PubChem Substance. Information regarding the pharmacological properties of these compounds was thus sought on PubMed, which is denoted in supplementary table 3.

For functional grouping of hit compounds, the dose–response curves for the six readouts of NK cell responses for all hits were sorted and visualized by a hierarchical clustering strategy with the R software (version 2.15.2, the R Core Team [2013]).

Results

Design of high-throughput screening assay for evaluation of primary human NK cell effector functions

To assess the effect of commonly used drugs on Fc-receptor-dependent NK cell responses, we performed a screen quantifying an open LFA-1 conformation (as an adhesion readout), MIP-1 β (as a readout of de novo protein expression), and CD107a (as a read-out of release of cytotoxic granules) after 1 h of cell stimulation. At this time point, these readouts provide sensitive and robust quantification of cytotoxic lymphocyte activation [16–19] TNF and IFN- γ , that represent additional readouts of cytotoxic lymphocyte function, were omitted as they are not reproducibly detected in NK cells until 3–5 h after stimulation [10, 20]. With respect to the screening procedure, a suitable donor

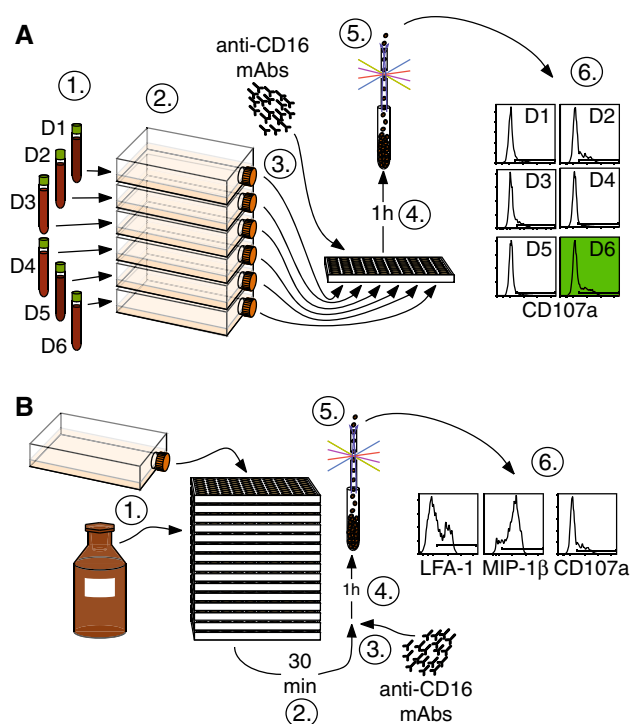


Fig. 1 Schematic illustration of the Prestwick Chemical Library screening assay. **a** Selection of a suitable donor based on pre-screening of NK cell responses. PBMCs were 1 isolated from peripheral blood of 6 donors and 2 incubated overnight at 37 °C. The cells were 3 distributed to wells of a 96-well plate followed by 4 the addition of anti-CD16 mAbs. The cells were 5 stimulated for 1 h at 37 °C, followed by 6 staining with fluorochrome-conjugated mAbs and flow cytometric analysis. 7 Based on analysis of flow cytometric data, one donor with a high percentage of exocytosing NK cells of total PBMC was selected for use in the compound screen. **b** Primary screening assay. PBMCs from a selected donor were 1 distributed into wells of a 96-well plate and 2 pre-incubated with compounds for 30 min at 37 °C. 3 Anti-CD16 mAbs was thereafter added to the cells, and 4 they were subsequently stimulated for 1 h at 37 °C, followed by 5 staining with fluorochrome-conjugated mAbs and flow cytometric analysis. Flow cytometric data were 6 analyzed with regard to readouts of all different cellular responses

having a high percentage of exocytosing NK cells was first identified among six donors through pre-screening (Fig. 1a). Thereafter, PBMC from the selected donor was pre-incubated with compounds from the chemical library or a positive (PP2) or negative (DMSO only) control and thereafter stimulated with anti-CD16 antibody for 1 h, followed by staining and flow cytometry (Fig. 1b).

Primary screen of Prestwick Chemical Library

To systematically evaluate the impact of pharmacological compounds on NK cell effector functions, the Prestwick Chemical Library was screened. In the primary screen, the highest/median/lowest plate Z' -factors were 0.89/0.74/0.24, 0.89/0.75/0.67, and 0.70/0.52/0.31, for

readouts of adhesion, chemokine production, and exocytosis, respectively (Supplementary table 1).

The data were normalized to controls on the respective plates. After normalization, the value for the solvent alone averaged 0 and that for the PP2 controls 100. Thus, inhibitors displayed positive values: inactive compounds displayed values close to 0, whereas enhancers displayed negative values. The data were non-normally distributed and contained a high rate of outliers (Fig. 2a). Hits were therefore identified using a quartile-based method, developed for hit selection in non-normally distributed screening data [12]. First, 103 active compounds (9 % of the library), defined as compounds that modulated any of the three readouts of NK cell responses above or beneath the respective quartile-based thresholds, were identified (Fig. 2a; Supplementary table 2). Second, ten compounds (10 % of the active compounds of which five were enhancers) that increased cell death were excluded. Third, 16 active fluorescent compounds (17 % of the non-toxic active compounds of which 15 were enhancers) that interfered with assay readouts were excluded based on increased signals in $CD3^+CD56^-$ T cells that do not typically respond to stimulation of CD16. The remaining 77 compounds were identified as hits in the primary screen (Fig. 2b; Supplementary table 2).

To confirm results from the primary screen, the selected 77 hit compounds were re-evaluated in a replicate screen (Fig. 2c). Compounds modulating at least one NK cell response more than three standard deviations compared with the solvent control were considered confirmed. With this threshold, nine compounds (12 % of the hit compounds; of which seven were enhancers) did not confirm their activity from the primary screen (Supplementary table 2). Of the 68 confirmed compounds, 56 were inhibitors and 12 were enhancers of NK cell responses (Fig. 2b). Several of the confirmed compounds affected multiple readouts (Fig. 2d, e). Of note, no compound significantly enhanced MIP-1β nor selectively inhibited LFA-1.

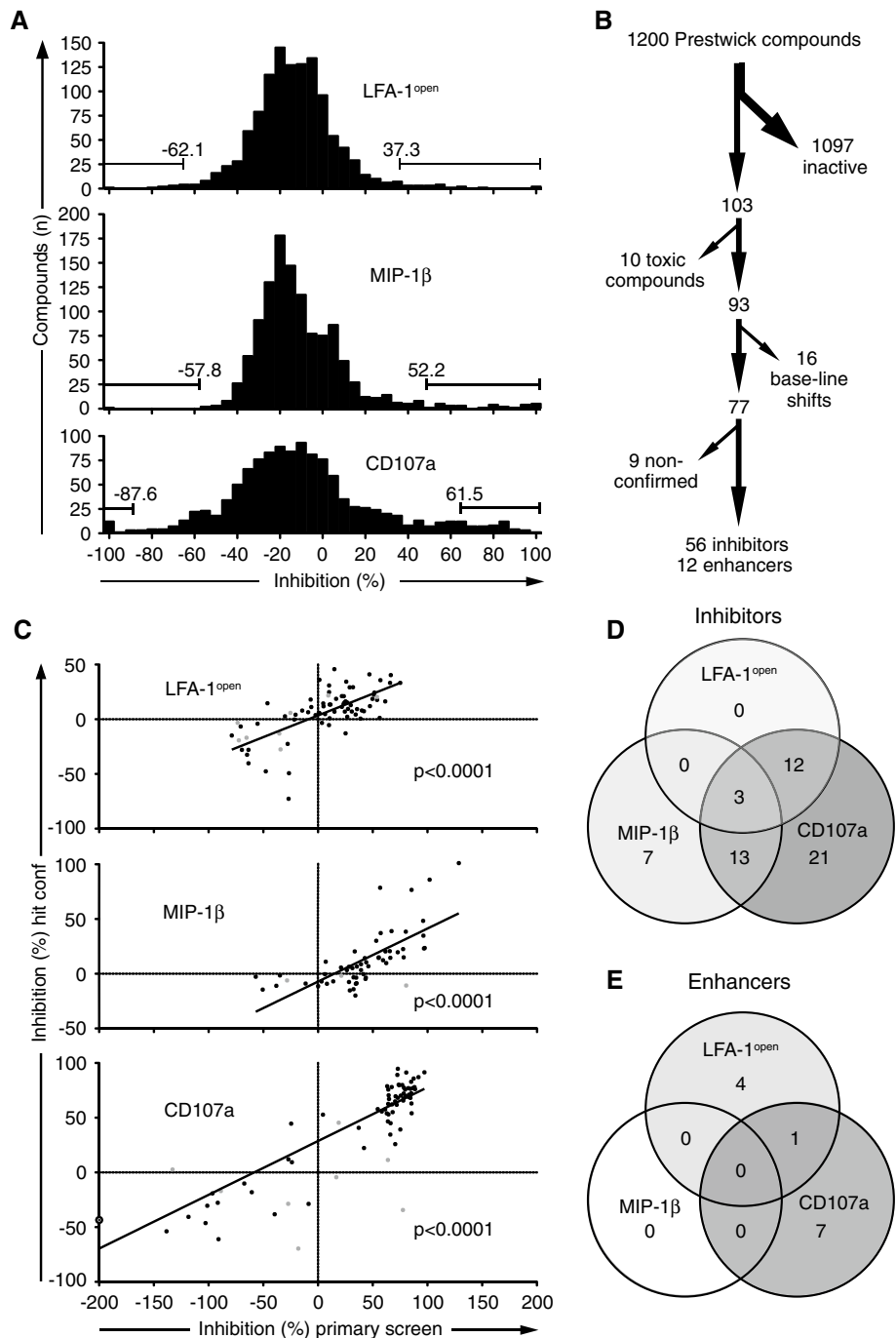
Structural clustering and pharmacological profiling of hits

The Prestwick Chemical Library contains compounds that share structural elements as well as pharmacological properties. In an attempt to stratify compounds in an unbiased manner, a hierarchical clustering strategy based on two-dimensional molecular fingerprints was used. This generated 88 clusters containing 2–166 compounds and 21 structurally unrelated compounds. Clusters proximal in the numerical order shared a higher degree of structural elements than distal clusters (Fig. 3a).

With this stratification, 20 of the 88 structural clusters contained hits (Fig. 3a; Supplementary table 3). In addition, one hit was a structurally distinct compound.

Fig. 2 Identification of compounds modulating NK cell activity from the Prestwick Chemical Library screen.

a Histogram of normalized data on readouts of NK cell activity. Bars indicate quartile-based selection thresholds for readouts of adhesion (LFA-1^{open}), chemokine production (MIP-1 β), and exocytosis (CD107a), as indicated. **b** Flow chart depicting the hit selection process, indicating the number of substances excluded in each step as well as the final number of hit compounds. **c** Comparison of primary screen and hit confirmation screen results for the 77 primary screening hits. Black dots represent the responses of confirmed hits, gray dots non-confirming compounds. For CD107a, the black and white dot on the Y axis indicate a compounds with -317% inhibition in the primary screen. The p-values were generated by linear regression analyses. **d**, **e** Venn diagrams depicting the overlap among inhibitory (**d**) and enhancing (**e**) activity on distinct readouts of adhesion, chemokine production, and exocytosis in the original screen

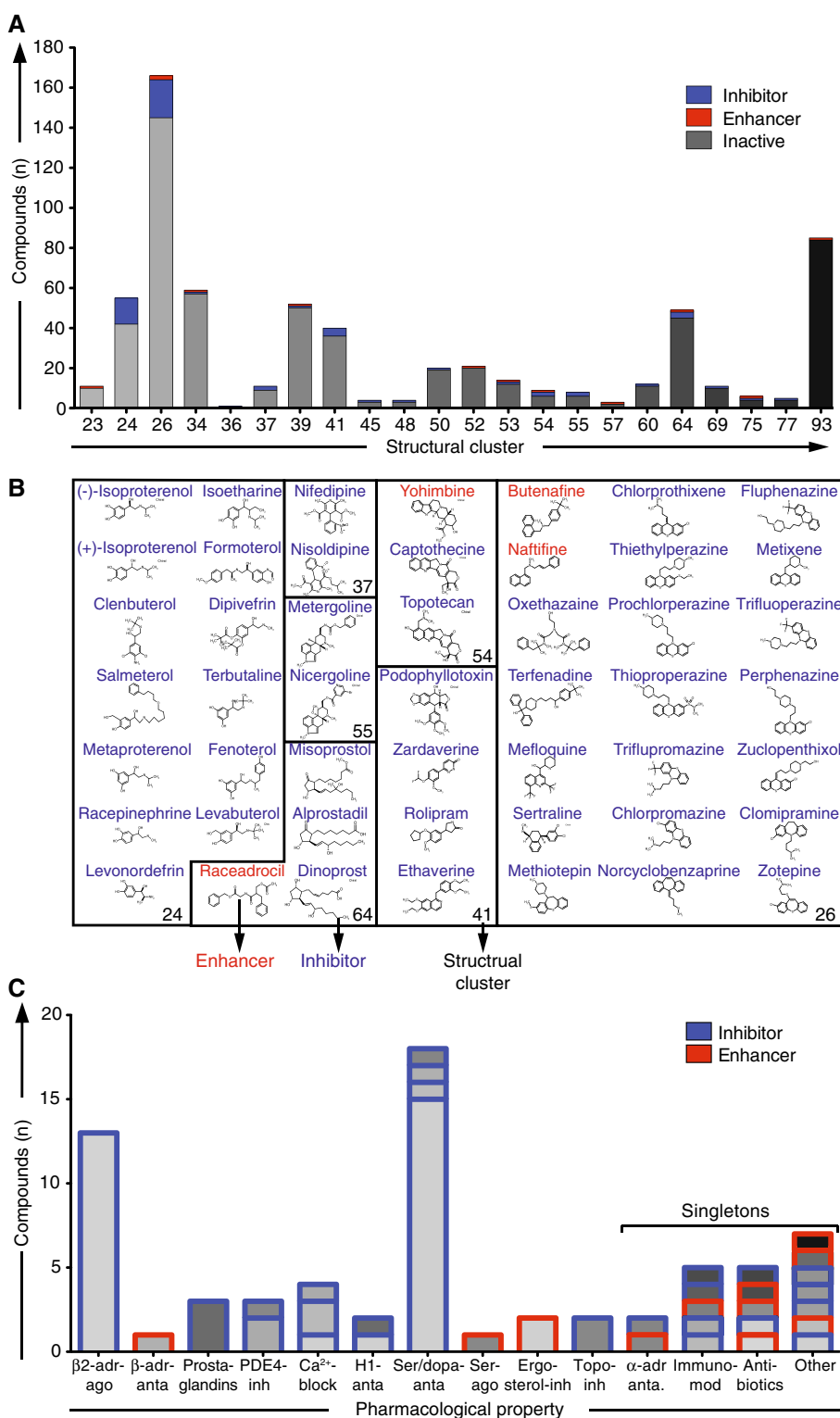


In total, 17 clusters contained inhibitors, of which seven structural clusters included more than one inhibitor. Furthermore, eleven clusters contained enhancers, of which only cluster 26 contained more than one enhancer. The compounds within some structural clusters also shared a common pharmacological profile, as exemplified by the β -phenylethanolamines within cluster 24, which act as β 2-adrenergic agonists, or the dihydropyridines within cluster 37, which act as L-type Ca^{2+} -channel blockers (Fig. 3b). However, there were examples of structural clusters in

which some members diverged in terms of the reported pharmacological profiles as well as our observed functional responses despite structural similarity. For example, structural clusters 26 and 64 both contained members with inhibitory as well as enhancing activities (Fig. 3b).

To address the shortcomings in terms of a direct translation between structural classification of compounds and functional responses in NK cells, we initiated a systematic examination of the reported pharmacological effects of the hits (Fig. 3c; Supplementary table 3). Based on

Fig. 3 Structural and pharmacological clustering of hits. **a** Molecular fingerprint-based clustering and stratification of the Prestwich Chemical Library. The graphs indicate the number of compounds within each cluster, as indicated. Only those clusters that contain hits are shown. Inhibitors (blue) and enhancers (red) are indicated. The intensity of black stain is proportional to the cluster number, giving cluster 1 white and cluster 110 black colors. **b** Two-dimensional structure of compounds within structural clusters with more than one hit. **c** Clustering of hit compounds based on reported pharmacological effects. Inhibitors (blue) and enhancers (red) are indicated. The intensity of the black stain is proportional to the structural cluster number



pharmacological information, the compounds could be categorized into eight major pharmacological profiles, namely $\beta 2$ -adrenergic agonists ($n = 13$), prostaglandins ($n = 3$), phosphodiesterase (PDE)4 inhibitors ($n = 3$), Ca^{2+} -channel blockers ($n = 4$), histamine H1-receptor antagonists

($n = 2$), serotonin/dopamine receptor antagonists ($n = 18$), topoisomerase inhibitors ($n = 2$), and ergosterol synthesis inhibitors ($n = 2$), among which all but ergosterol synthesis inhibitors were inhibitory on the NK cell responses. Eleven inhibitors and ten enhancers that were structurally

and pharmacologically unique were also identified. Two represented structurally diverse α -adrenergic antagonists (one inhibitor and one enhancer). Moreover, five of these compounds had reported immunomodulatory and five antibiotic/antifungal properties. Notably, among the remaining compounds, timolol, a β 2-adrenergic antagonist, and quipazine, a serotonin receptor antagonist, were identified as enhancers. Thus, 45 or 80 % of the inhibitors could be classified as belonging to one of seven pharmacological classes, whereas 2 or 17 % of the enhancers were related in both structure and pharmacological profile.

Dose–response curves of pharmacological classes

To determine whether the pharmacological classes possessed distinct modulatory patterns and consistent activity among donors, dose–response experiments were performed. Here, to also evaluate the impact of the hit compounds on the production of key cytokines produced by NK cells, the cells were stimulated with an anti-CD16 antibody for 4 h. PBMC from three selected donors were pre-incubated for 30 min with DMSO only, 5 μ M PP2, or hit compounds at five concentrations ranging from 0.04 to 25 μ M. Thereafter, the cells were stimulated, stained, and analyzed by flow cytometry. Surface expression of CD107a and the LFA-1 conformation-specific epitope as well as intracellular expression of MIP-1 β were examined. In addition, to gain further insight into the modulation of NK cell functional capacities, intracellular expression of IFN- γ , TNF, and CD69 was also evaluated.

The β 2-adrenergic agonists displayed pronounced inhibition of exocytosis, TNF, and IFN- γ production at all evaluated concentrations (Fig. 4). Notably, the β 2-adrenergic antagonist timolol specifically enhanced degranulation (Fig. 4). Among the prostaglandins, prostaglandin E1 produced inhibition curves similar to those of the β 2-adrenergic agonists, whereas prostaglandin F2 α and the synthetic prostaglandin E1 analog, misoprostol, were less potent. The PDE4 inhibitors induced a pattern of inhibition much like the β 2-adrenergic agonists and prostaglandins, mostly affecting exocytosis, TNF, and IFN- γ production, to a lesser extent LFA-1, but with remarkably little effect on MIP-1 β and CD69 expression. The Ca²⁺-channel blockers acted at high concentrations, inhibiting exocytosis, TNF, and IFN- γ production. In contrast, the histamine H1-receptor antagonists at high concentrations inhibited exocytosis, TNF, and IFN- γ production as well as MIP-1 β and CD69, but had little effect on LFA-1. The group of serotonin/dopamine receptor antagonists displayed dose–response curves similar to those produced by the histamine H1-receptor antagonists. Notably, ergosterol synthesis inhibitors specifically enhanced exocytosis and displayed some inhibition of TNF and IFN- γ production. As expected, topoisomerase

inhibitors inhibited TNF and IFN- γ and, to some extent, CD69 synthesis. However, interestingly, MIP-1 β synthesis was not affected. In summary, the classification of compounds based on pharmacological profiles revealed concerted modulation of NK cell responses within different pharmacological classes as well as consistent dose–response patterns among multiple donors.

Ability of hit compounds to induce response-specific or threshold-controlled NK cell inhibition or activation

Modulatory compounds may act either globally by broadly inhibiting or enhancing NK cell signaling or specifically on pathways that control distinct responses. Previously, we described a hierarchy among NK cell responses, with induction of TNF and IFN- γ production requiring stronger stimuli than those required for induction of LFA-1, MIP-1 β , and CD107a [19]. To probe the modulation of hit compounds with respect to the different NK cell responses, pairwise comparisons of compound activity on different readouts of activation were plotted (Fig. 5). Since threshold effects for inhibition of CD107a, TNF, and IFN- γ versus LFA-1 or MIP-1 β were evident, the plots suggested that compounds may act by selectively affecting responses requiring the highest degree of activation signaling. Moreover, a strong correlation between the inhibition of TNF and that of IFN- γ indicated very similar signaling requirements for the induction of TNF and IFN- γ production. Notably, data also indicated a threshold for inhibition of CD69 similar to that of MIP-1 β (Fig. 5). Supporting this notion, with compounds at 25 μ M, hierarchical clustering of responses demonstrated the close relationship between inhibition of MIP-1 β and CD69 responses, as well as that of TNF and IFN- γ (Supplementary figure 2). Of note, some data points did not follow this general trend, indicating that certain compounds act more selectively on distinct responses.

To functionally relate all hit compounds and identify compounds selectively affecting specific NK cell responses, hierarchical clustering based on the activity of hit compounds on multiple readouts of NK cell function was performed (Fig. 6). This stratification revealed three major functional groups. The first major functional group inhibited CD107a, TNF, and IFN- γ as well as MIP-1 β , and CD69 at high concentrations. This group encompassed all 19 inhibitory compounds within structural cluster 26. This group consisted pharmacologically of serotonin/dopamine receptor antagonists, the two histamine H1-receptor antagonists identified in the screen, as well as two of the Ca²⁺-channel blockers. Of note, the group also contained structurally and pharmacologically unique compounds, encompassing parthenolide (NF κ B inhibitor), perhexiline (carnitine palmitoyl transferase inhibitor), and nicergoline (α 1-adrenergic antagonist). A second major functional

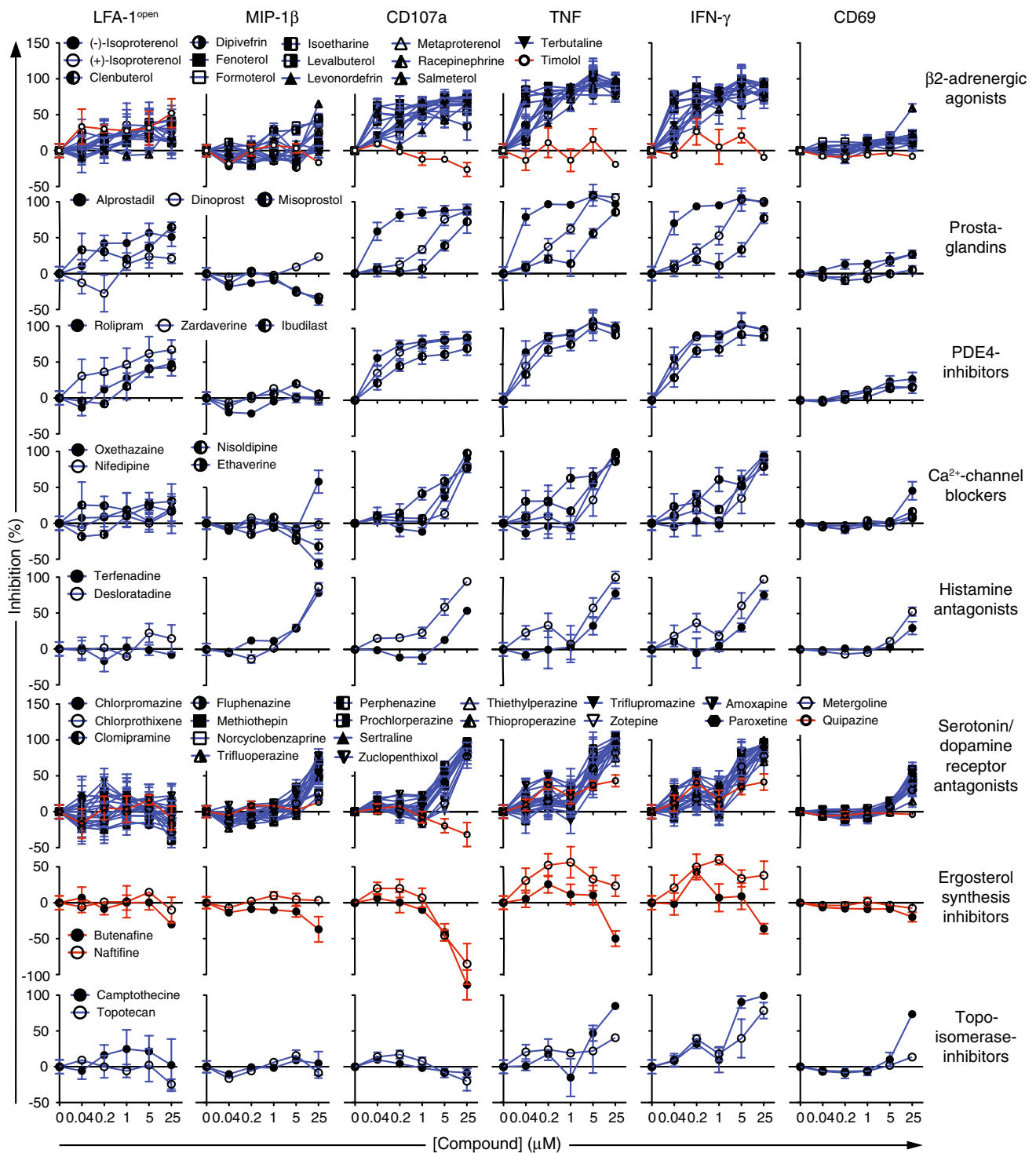


Fig. 4 Dose–response curves of selected hit compounds on multiple readouts of NK cell activity. Freshly isolated, resting PBMC from three donors were pre-incubated with hit compounds at various concentrations for 30 min, as indicated. The cells were stimulated with anti-CD16 mAb, in the presence of compounds, for 4 h followed by staining with fluorochrome-conjugated mAbs and flow cytomet-

ric analysis. *Graphs* depict the activity of compounds from different pharmacological clusters on multiple readouts of NK cell function, as indicated. *Graphs* show inhibition relative to compound concentrations, as indicated. Values indicate mean compound activity of three donors. *Error bars* indicate the standard deviation

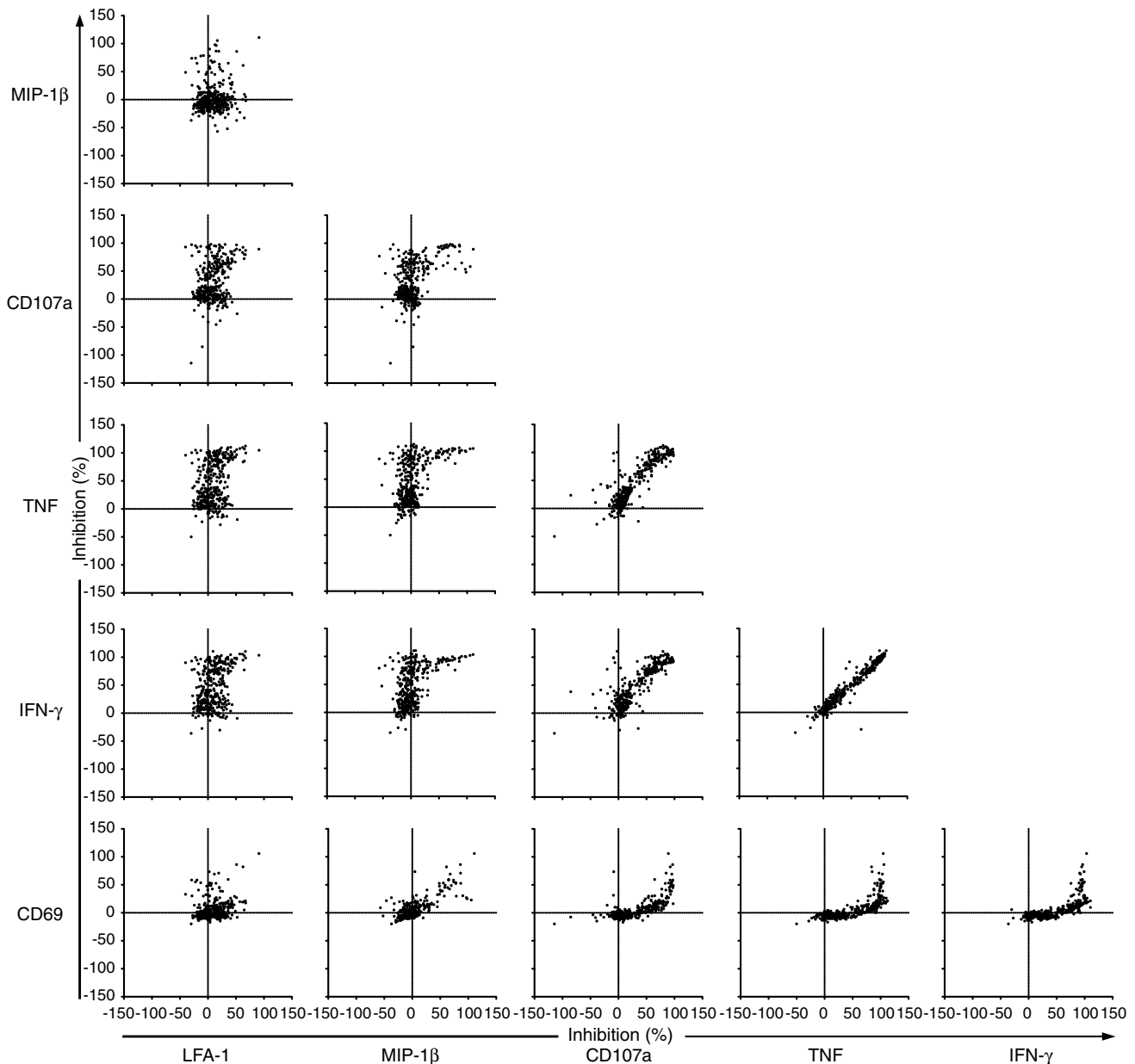


Fig. 5 Comparison of the impact of compounds on various functional NK cell readouts. Freshly isolated, resting PBMC from three donors were pre-incubated with hit compounds at various concentrations for 30 min, as indicated. The cells were stimulated with anti-

CD16 mAb, in the presence of compounds, for 4 h followed by staining with fluorochrome-conjugated mAbs and flow cytometric analysis. *Graphs* show pairwise comparisons of relative inhibition by various compounds for functional readouts, as indicated

group, inhibiting mainly CD107a, TNF, and IFN- γ , contained eight compounds from structural cluster 24. In addition to these β 2-adrenergic agonists, this group also contained two prostaglandins, one PDE4 inhibitor, two Ca^{2+} -channel blockers, as well as moricizine (Na^{+} -channel blocker). A third major functional group, strongly inhibiting CD107a, TNF, and IFN- γ , but also affecting LFA-1, contained five compounds from structural cluster 24. In addition to these β 2-adrenergic agonists, this third group also contained one prostaglandin, two structurally related

PDE4 inhibitors, and trazolate [γ -aminobutyric acid (GABA)-A receptor inhibitor]. Finally, two enhancers from structural cluster 26, both ergosterol synthesis inhibitors, belonged to the same group. The remaining compounds were structurally and pharmacologically unique and had varying activities on readouts of NK cell function. Of these, six were inhibitors and ten enhancers. The six inhibitors included two topoisomerase inhibitors, the calcineurin inhibitor cyclosporine A, the protein synthesis inhibitor cycloheximide, the antibiotic lincomycin, as well

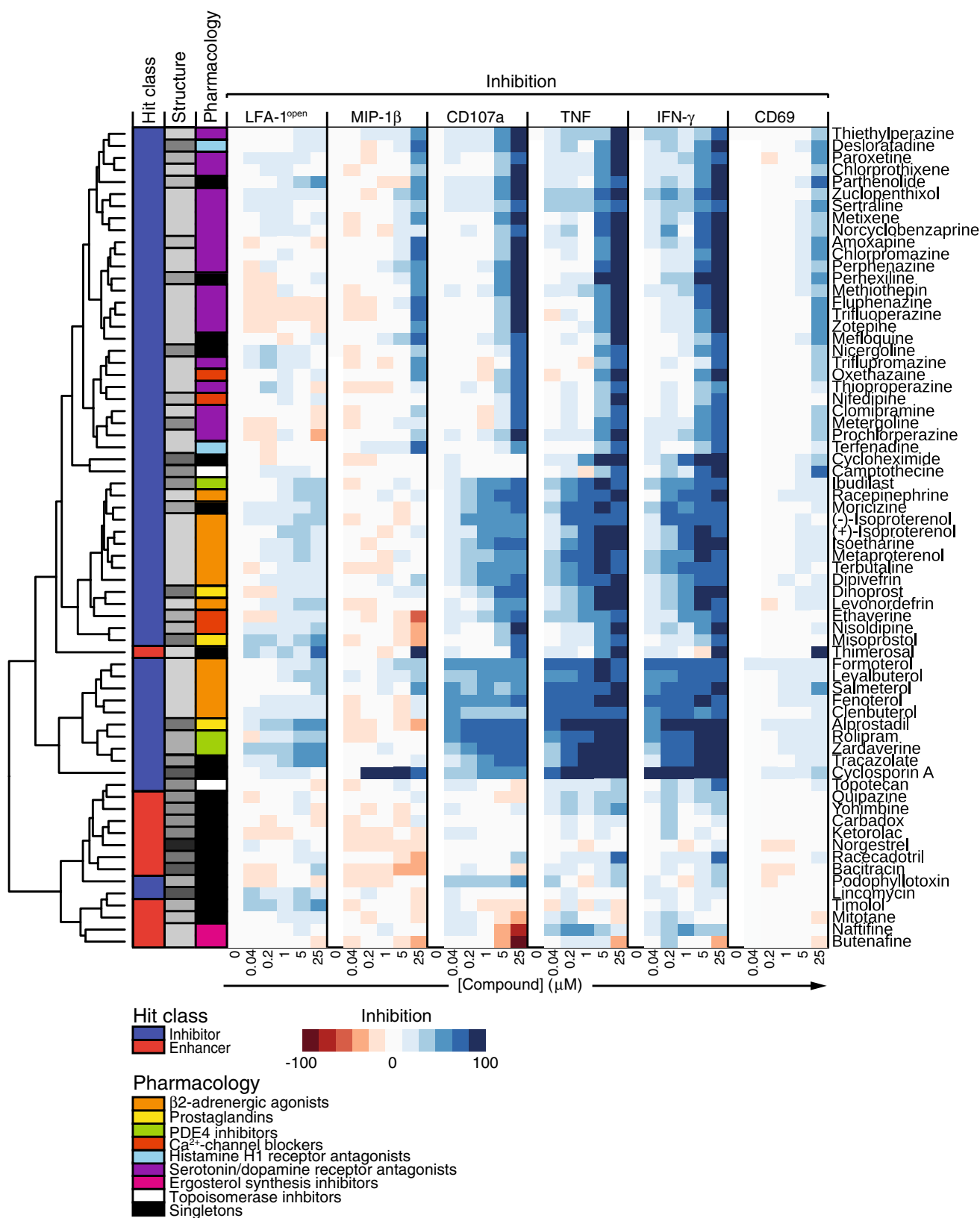


Fig. 6 Functional clustering of hit compounds. Freshly isolated, resting PBMC from three donors were pre-incubated with hit compounds at various concentrations for 30 min, as indicated. The cells were stimulated with anti-CD16 mAb, in the presence of compounds, for 4 h followed by staining with fluorochrome-conjugated mAbs

and flow cytometric analysis. All hit compounds from the Prestwich Chemical Library were hierarchically clustered based on their dose-response inhibition of multiple readouts of NK cell function. Heatmaps indicate the inhibition of specific readouts for each individual compound relative to compound concentrations, as indicated

as the microtubule assembly inhibitor podophyllotoxin. Interestingly, podophyllotoxin selectively inhibited NK cell exocytosis. The majority of the enhancers identified in the 1-h screen demonstrated little activity at the 4-h time point.

Discussion

Here, we have developed and assessed a high-throughput drug screening assay for identification of commonly used drugs that affect Fc-receptor-dependent signaling for NK cell activation. A diverse library consisting of 1,200 in-use or previously approved drugs, making up a large fraction of the Western world's Pharmacopoeia, was studied. Sixty-eight potent inhibitors and enhancers of adhesion, chemokine production, or exocytosis were defined as hit compounds. Evaluation of multiple independent donors revealed reproducible effects of the hit compounds on all readouts of NK cell activation. In follow-up studies, the hits were examined according to similarities with respect to structural elements, reported pharmacological profiles, and activity on multiple readouts of NK cell responses. Although our screen does not cover the complete pharmacopoeia, does not encompass compound metabolites, and does not detect compounds with slow-acting effects, our results do provide a resource for understanding the effect of most commonly used drugs on NK cell functions.

The Prestwick Chemical Library was recently screened for compounds that affected exocytosis by the TALL-104 T-cell line in response to the stimuli phorbol-12-myristate-13-acetate and thapsigargin, which represent a synthetic protein kinase C agonist and an inducer of intracellular Ca^{2+} mobilization, respectively [21]. In that screen, flow cytometric evaluation of induced CD107a surface expression identified 24 hits. Eight of those 24 hits were also identified in our screen. Of the eight compounds, we excluded three due to toxicity or base-line shifts. Notably, the screen by Florian and colleagues did not identify hits representing β 2-adrenergic agonists, prostaglandins, PDE4 inhibitors, Ca^{2+} -channel blockers, or ergosterol synthesis inhibitors [21]. Thus, screens aimed at identifying clinically relevant pharmacological effects necessitate the inclusion of primary cells as well as physiological stimuli. Our discussion will focus on the classes of drugs that had strong effects on NK cell responses, although several drugs used in cancer therapy, contained within our screen but not reaching the hit cutoff, may also be of interest.

Through the use of hierarchical clustering based on the observed functional activities, three major groups of inhibitors were defined. Two groups contained compounds that inhibited adhesion, exocytosis as well as TNF and IFN- γ production with different potencies. These two functional groups contained three major classes of compounds based

on pharmacological profiling: β 2-adrenergic agonists, prostaglandins, and PDE4 inhibitors. Nine inhibitors were β 2-adrenergic agonists (either selective or non-specific for the β 2-adrenoreceptor). These compounds are mainly used as bronchodilators in patients with asthma, but also to delay childbirth. The β 2-adrenoreceptor is G_s -protein coupled, with cAMP as a second messenger. There are multiple reports supporting the role of β 2-adrenergic agonists in inhibiting NK cell and CTL cytotoxicity and transcription of cytokines [22, 23]. Three inhibitors were prostaglandins, which are used for the induction of childbirth, prevention of gastric ulcers, and for treatment of sexual dysfunction. Prostaglandins have previously been shown to potently inhibit NK cell cytotoxicity [24] and, like β 2-adrenergic agonists, stimulate cAMP synthesis. Four inhibitors were PDE4 inhibitors, which are used for treatment of asthma and are also being evaluated for efficacy in other inflammatory conditions. PDEs enzymatically degrade cAMP; consequently, the inhibition of PDE results in increased intracellular cAMP levels. PDE4 inhibitors have previously been shown to inhibit NK cell cytotoxicity as well as T-cell cytokine production [24, 25]. Thus, β 2-adrenergic agonists, prostaglandins, and PDE4 inhibitors likely all act through increasing intracellular cAMP levels. Notably, comparison of our data on the potency of β 2-adrenergic agonists for inhibition of degranulation at 10 μM with that of another screen quantifying the potency of β 2-adrenergic agonists for induction of cAMP revealed a strong positive correlation (Supplementary figure 3) [26]. In T cells, increased cAMP promotes protein kinase A-dependent phosphorylation and the activity of C-terminal SRC kinase, which in turn phosphorylates and suppresses SRC kinase activity and T-cell receptor signaling [27]. This mechanism may explain the inhibition of exocytosis, cytokine production, as well as adhesion. Interestingly, the reasons why cAMP signaling does not affect the induction of MIP-1 β and CD69 within the time frame investigated in this study remain to be elucidated. The clinical effects of potent inhibitors from this group are being further investigated.

A third major group of inhibitors identified by hierarchical clustering based on the functional activity of hit compounds consisted of serotonin/dopamine receptor antagonists, a few compounds with more selective serotonin receptor antagonism or serotonin reuptake inhibitors, as well as two histamine H1-receptor antagonists. A majority of the serotonin/dopamine receptor antagonists were phenothiazines and their derivatives, with antiemetic and/or neuroleptic activity. Serotonin receptor antagonists or reuptake inhibitors are mainly used for their anti-depressive properties, whereas anti-histamines are used to treat allergies. Distinct from the other major functional groups, these compounds inhibited exocytosis and expression of chemokines, cytokines, as well as CD69, but only at high

concentrations. Both serotonin and dopamine were represented in our screen. Serotonin lacked activity, whereas dopamine was a modest inhibitor. Serotonin transporters may be expressed in lymphocytes [28], and tricyclic antidepressants have been reported to inhibit NK cell cytotoxicity [29]. Expression of dopamine receptors has also been found in NK cells [30]. Dopamine can be produced by regulatory T cells and dendritic cells and thereby modifies T-cell function [31]. Alternatively, phenothiazines may inhibit lymphocyte activation by antagonizing calmodulin-dependent signaling [32]. Furthermore, NK cells reportedly express histamine H₂- and H₄-receptors, which facilitate NK cell chemotaxis, and histamine may enhance NK cell-mediated cytotoxicity in a monocyte-dependent manner [33, 34]. Thus, serotonin, dopamine, and histamine can modulate immune cell function. In our assay, the endogenous sources of serotonin, dopamine, and histamine are not known; therefore, how receptor antagonists in this setting inhibit NK cell function is not clear. It is, however, possible that such antidepressants and antipsychotics might modulate immune cell function in patients.

Besides the major groups based on similarities in modulation of NK cell responses, four compounds with described L-type Ca²⁺-channel blocking activity displayed a similar capacity to inhibit exocytosis and cytokine production at high concentrations, but differed with regard to their effects on production of MIP-1 β . Three of these Ca²⁺-channel blockers are used for the treatment of hypertension, whereas one (oxethazine) is used as a local anesthetic and may also possess sodium-channel-blocking activity. Extracellular Ca²⁺-influx is required for NK cell activation and mutations in the Ca²⁺ release-activated Ca²⁺-channel ORAI1 abrogates exocytosis and cytokine production by NK cells [35]. The role of an L-type Ca²⁺-channel in NK cell activation is less clear, but nifedipine was previously shown to inhibit NK cell cytotoxicity by up to 50 % [36]. Moreover, cyclosporin A, a calcineurin inhibitor [37], is one of the most potent inhibitors and is used to treat graft versus host reactions as well as rheumatic diseases. Cyclosporin A was the only compound that showed strong inhibition of MIP-1 β at low concentrations and also displayed pronounced inhibition of exocytosis. The inhibitory capacity of cyclosporin A on NK cells function is well documented [38]. As expected, the topoisomerase inhibitors camptothecin and topotecan as well as the translation inhibitor cycloheximide were identified as potent inhibitors of cytokine production. Of note, podophyllotoxin was identified as a selective inhibitor of exocytosis. Podophyllotoxin is used for treatment of human papilloma virus-caused warts and binds to microtubuli, thereby inhibiting mitosis [39, 40]. How podophyllotoxin specifically inhibits NK cell exocytosis is of interest for further investigation.

Of the enhancers, the two anti-fungals naftifine and butenafine were the only structurally and functionally related compounds. These compounds are used for topical treatment of fungal infections and have been identified as ergosterol synthesis inhibitors (an enzyme required for synthesis of cell walls in fungi). However, their mechanism of action on fungi is disputed [41, 42]. Remarkably, naftifine and butenafine selectively enhanced NK cell exocytosis. How these compounds act on lymphocytes is not clear, but they are reported to bind lipids and affect membrane integrity [42]. Notably, these compounds can cause contact dermatitis reactions after skin application [43], suggesting immunomodulatory effects besides their possible direct action on fungi. In this context, NK cells have been linked to the development of contact dermatitis [44, 45]. Provided they can safely be administered, naftifine and butenafine may be of interest in regard to enhancing the effect of ADCC in antibody therapy.

It is notable that a majority of hit compounds modulated NK cell responses in a manner reflecting the different thresholds for induction of specific responses. For example, inhibitory compounds generally affected IFN- γ and TNF production more severely than LFA-1 activation and degranulation, corresponding to higher levels of activation required for induction of cytokine gene expression (21). Selective inhibition of NK cell responses may have clinical implications. Cytokine production is central in coordinating antiviral defenses. Thus, some drugs may not significantly impact the ability of NK cells to eliminate infected cells; they may still impair immune responses by hampering cytokine signaling networks.

Our assay can be used for screening other compound libraries. In our experience, the sensitivity of readouts such as LFA-1, MIP-1 β , and CD107a is high following 1 h of stimulation. However, the induction of cytokines such as IFN- γ and TNF can be evaluated in longer assays. In such assays, though, no modulation of responses requiring a low threshold for activation, e.g., LFA-1 and MIP-1 β , may occur. Therefore, studying multiple time-points may provide important information with regard to targeted signaling pathways. Moreover, technical advancements, e.g., using novel flow cytometers to study more parameters [46, 47], may further improve analyses with respect to defining cellular populations in greater detail, facilitating investigation of additional functional and signaling parameters, and allowing multiplexing of samples.

In summary, the results document a robust high-throughput flow cytometric assay for evaluating of the effects of compounds on multiple NK cell responses. This comprehensive review of in-use pharmacological compounds details functional outcomes from their interactions with primary NK cells. These results highlight major clusters of active compounds with similar mechanisms of action and

reveal specific compounds whose unusual functional properties invite further exploration, particularly with respect to their possible role in modulating ADCC. Clinically, this work reveals the extent and some possible mechanisms for immunomodulatory effects or side effects to commonly used drugs. These insights may thereby facilitate new uses of such drugs for immunomodulatory purposes as well as aid in predicting of the properties of new compounds, not only on NK cells but more widely with respect to cytotoxic lymphocyte subsets.

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