Published in final edited form as:

Medchemcomm. 2013 January 1; 4(1): . doi:10.1039/C2MD20073B.

A Mast Cell Degranulation Screening Assay for the Identification of Novel Mast Cell Activating Agents

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Abstract

The development and use of vaccines and their ability to prevent infection/disease is a shining example of the benefit of biomedical research. Modern vaccines often utilize subunit immunogens that exhibit minimal immunogenicity and require the use of adjuvants to maximize the induction of protective immune responses. We recently described a novel class of vaccine adjuvants, mast cell (MC) activators, that exhibit safe and effective vaccine adjuvant activity when administered by intranasal or intradermal routes. A compound library containing 580 functionalized benzopyrans, a structural motif found in a diverse array of natural and designed bioactive compounds, was screened using a MC degranulation assay to identify novel MC activating compounds for future evaluation as novel vaccine adjuvants. This approach identified 12 novel MC degranulating compounds. Therefore, MC degranulation can be used to reliably detect novel compounds for evaluation as adjuvants for use in mucosal vaccine strategies.

Introduction

The emergence of new infectious agents, as well as current biological threats has created a great need for the rapid development of safe and efficacious vaccines. However, current strategies to create such vaccines are unable to meet the need for broad prophylactic immune protection, especially along mucosal portals such as the lung or gastrointestinal tract used by pathogens to gain access to deeper tissues. A defect in these strategies is a lack of effective adjuvant compounds, the immuno-stimulatory component of vaccines that are able to evoke protective immune responses along mucosal sites. For instance, alum, one of the few

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licensed adjuvants for human use, is only effective in the induction of systemic immune responses.¹⁻⁷ Therefore, there is a need to identify new mucosal adjuvant compounds that are effective, safe and economical for future vaccine design.

Recently, several studies have elucidated a role for the mast cell (MC), which are granulated tissue resident leukocytes in the development of adaptive immunity. MC release of pro-inflammatory mediators such as TNF or IL-1 were found to affect the development of both humoral and cellular components of immunity in mice after challenge with diverse stimuli including bacteria, the TLR7 ligand imiquimod or contact sensitizers such as FITC.⁸⁻¹⁰ Moreover, targeted activation of MCs with the small molecule MC activator compound 48/80 (c48/80) was found to induce safe and robust systemic and mucosal immune responses against various protein antigens when co-administered with vaccine antigens at mucosal surfaces.^{11,12} In all cases, MCs were suggested to affect adaptive immunity by enhancing the recruitment of dendritic cells (DCs) and lymphocytes into local lymphoid tissues, a prerequisite for optimal immune responses. These data suggest that MCs may be a common denominator in the modulation of immunity to many diverse agents and molecules capable of activating MC are potentially highly effective adjuvants. Therefore, MCs might be used as sensors to screen chemical libraries to detect novel compounds with mucosal adjuvant activity.

In this study, we describe the development of a novel screen using the MC degranulation response as a sensitive monitor to detect potential mucosal adjuvant compounds. We report that this methodology successfully detected 12 novel MC degranulating compounds from a library containing 580 functionalized benzopyrans, a privileged structural motif found in a diverse array of natural and designed bioactive compounds.¹³⁻¹⁸ Therefore, MC degranulation can be used to reliably detect novel compounds which may greatly expand our current lexicon of potential adjuvant compounds for use in mucosal vaccine strategies.

Results

Development of an in vitro mucosal adjuvant screen based on MC activation

A scheme for the use of readily available MC lines to screen for novel mucosal adjuvant compounds is outlined in Figure 1. For this screening assay, we selected the mouse mast cell line MC/9.¹⁹⁻²⁴ The MC/9 mouse mast cell line expresses IgE Fc receptors and degranulates and produces cytokines in response to IgE-antigen stimulation.²⁵ The MC/9 mouse mast cell line has been described as a cell line representative of a “mucosal mast cell”.^{22, 25} The MC/9 cell line was specifically selected for our screening assay because they are not readily activated by agonists and therefore would minimize false-positive reactions. Indeed, others have reported that stimulation of MC/9 cells for 15 minutes with 0.5 µg/ml of compound 48/80 induced minimal degranulation of MC/9 cells based on β-hexosaminidase release (1.5% of total) and tryptase (1.2% of total) while primary peritoneal mast cells released 16% of total β-hexosaminidase and 3.5% total tryptase when stimulated with 0.5 µg/ml compound 48/80.^{26, 27}

In this scheme, chemical compounds are mixed with the murine MC/9 MC line,¹⁹⁻²⁴ and evaluated for their ability to induce the release of MC granule contents, a process called degranulation. Degranulation, a reliable marker of MC activation, is assayed by examining cell culture supernatants for the presence of β-hexosaminidase (β-hex), an enzyme located in MC granules. Release of this enzyme implies that MCs have released their granule components, whereas its absence suggests that the chemical compound does not have the ability to activate MCs. To perform this assay, we modified a procedure described by others.^{26, 28} MC/9 cells were aliquotted in sterile Tyrode's buffer and added to each well of a sterile V-bottom plate at 5×10⁵ cells per well in a 100µl volume. For each 96-well plate,

internal plate controls included MCs treated with the positive control degranulating agent compound 48/80 (c48/80; doses of 49.6, 198.3 and 793.5 μ M corresponding to 31.25, 125 and 500 μ g/ml). C48/80 is a cationic mast cell stimulating chemical compound first reported for its ability to induce histamine secretion²⁹. Compound 48/80 appears to activate mast cells by a receptor-independent mechanism that activates G-proteins required for mast cell exocytosis.³⁰⁻³⁵ Other studies, however, suggest that Mas-related gene (Mrg) receptors on mast cells are involved in the mast cell activating activity of compound 48/80.³⁶ Additional assay controls included a vehicle control (DMSO) to evaluate background β -hex release and a maximum β -hex release control, which is cells treated with the detergent Triton X-100. Potential adjuvant compounds are added to each well in 100 μ l at a final concentration of 100 μ M.

Quality control results for the MC activating screen

An important component of this assay is the development of quality control criteria ensuring the reproducibility of the assay between multiple plates. Such reproducibility is required to establish a rank summary of the chemical compounds and their ability to induce β -hex release from MC/9 cells. These quality control criteria were established through iterative testing and determined to be: 1) baseline β -hex activity in vehicle control wells (spontaneous release measurements) should be consistent and below 0.2; 2) maximum β -hex activity in Triton X-100 wells (or maximum release measurements) should be consistent and above 1.0; and 3) that the calculated β -hex release from c48/80 treated samples should be consistent between assays with results varying less than 3 standard deviations from the mean. Results from plates that do not meet these quality control criteria are discarded and repeated. Using these quality control criteria, we evaluated MC activating capabilities of 580 functionalized benzopyrans which were prepared in accordance to the protocols developed by Nicolaou and co-workers.¹³⁻¹⁵ More specifically, this "benzopyran library" shared a common 2,2-dimethylbenzopyran core structure, which was synthesized through a Claisen rearrangements of hydroxy benzaldehyde derived propargyl ethers followed by further functionalization. In this manner, a diverse array of haloaryls, ethers, esters, amide, sulfonates and heterocycles could be readily accessed with high chemical yield and purity (Figure 2).

The compounds were examined for their ability to induce β -hex release from MC/9 cells in a total of 17 plates. As shown in Figure 3, our quality control results from these plates generated consistent minimal background β -hex release (average mean = 0.096 ± 0.02) (Figure 3A) and maximal β -hex release measurements (average mean = 2.26 ± 0.50) (Figure 3A) which are well within the quality control limits. Moreover, we found that treatment of MC/9 cells with the positive control mast cell degranulator c48/80 generated consistent results within three standard deviations from the mean of all assays (Figure 3B). Collectively, these data demonstrate the reproducibility of the assay and that a result from any given plate should be comparable with results on a separate plate.

Detection of novel adjuvant compounds using the MC activating screen

Using these quality control criteria, we found that our screen was able to detect 12 compounds (2%) of the total chemical library that were able to induce at least 5% release of β -hex from MC/9 cells. The results from the top 50 compounds are listed in Table 1. Of these, only compound number **10** induced greater than 10% β -hex release from MC/9 cells, while the rest induced β -hex release from MC/9 cells in a range between 5.5-8.4%. Intriguingly, structure-activity-relationship analysis of these compounds revealed three notable chemical classes that induce MC degranulating capability (Figure 4).

Structural analysis of the benzopyran library in correlation to MC activation

Recognizing the benzopyrans are privileged structures reported to illicit a wide-spectrum of biological responses, the structural diversity represented by the benzopyran library described herein was a crucial selection criteria at the outset of this study to maximize the likelihood of identifying a MC activator. Indeed, careful assessment of the MC activation screen revealed benzylamine, cyanostilbene and chalcone as the three structural classes of interest and which warrants further discussion and future investigation (Figure 4). Furthermore, preliminary structure-activity-relationship could be established within each structural class (Figure 5-7), which proved path-pointing for further chemical modifications of lead candidates to improve their MC activation capabilities.

- a. The benzylamines represents the major sub-group within the top 50 MC activating compounds (34%, 17 compounds) with compound **10** and compound **360** the most active members of this family. Further structural analysis of compound **10** suggested the necessity of its benzyl ether and secondary benzyl amine bearing an unsubstituted methylene linker, since compounds **287**, **147**, and **16**, **17**, **578** and **580** all showed significant loss of MC activation capability. For lead compound **360**, both the para-methoxy benzamide (cf. compounds **358** and **359**) and the indanyl substitutions (cf. compounds **181** and **227**) appear to be critical, where the carefully balanced steric environment is likely to be responsible for the observed MC activation capability (Figure 5).
- b. The cyanostilbenes represents 14% (7 compounds) of the top 50 MC activating compounds with compound **272** being the most active member of this family (Figure 6). Structure-activity analysis indicated that the regiochemistry of the cyanostilbene substitution was crucial for the observed activity (cf. compound **349**), together with marginal tolerability in the substitution of the two aromatic systems (cf. compounds **7**, **32**, **36** and **37**).
- c. Lastly, the chalcone family of compounds within the benzopyran library also displayed MC activation property that warrants additional attention (Figure 7). This class appeared to demonstrate a wider structure-activity window, in which compounds **293**, **309**, **479** and **290** all exhibited comparable potencies. Substitution at the non-benzopyran bearing aromatic ring appears to be an essential element for the observed activity (cf. compound **2**), and a variety of substituents including but not limited to halogen, hydroxyl, methoxy and methylmercaptan (cf. compounds **293**, **309**, **290** and **289**) were well tolerated. Interestingly, the chalcone olefin could be replaced with an analogous conformation restricting pyrazole functionality with good retention of MC activation capability (cf. compound **479**), suggestive of suitable functional handles at this location could be used to further improve the potency and pharmacological properties of this family of compounds.

Finally, the major MC activating compounds shortlisted and discussed herein showed little cross-structural resemblance. Although we have demonstrated the utility of the benzopyran library in the bioassay-guided identification of bioactives, it was not possible to formulate a unified structure-activity-relationship proposal. Nevertheless, the structural diversity exhibited by benzylamine, cyanostilbene and chalcone presents new avenues for further structural modifications and biological investigations.

Discussion

The development of new vaccines has long centered on the development of vaccine antigens that would appropriately target the pathogen of interest. However, with the development of vaccine regimens that use peptides or purified proteins as antigens has come the realization

that new adjuvant compounds, the immuno-stimulatory components of vaccines, must also be developed to fully realize the potency of different vaccines. Currently, many potential vaccines are limited by the dearth of potent adjuvants, both in terms of effectiveness and in the induction of protective antibody responses along mucosal surfaces. For instance, vaccine regimens against hepatitis B virus do not confer effective immuno-protection in all adults. This vaccine and many others use alum, one of only two approved adjuvants for use in the United States (the other is ASO4, a combination of the adjuvants alum and monophosphoryl Lipid A (MPL), a detoxified derivative of lipopolysaccharide from gram negative organisms). Therefore, the inability of these adjuvants to confer widespread protection, especially along mucosal surfaces suggests that the adjuvant repertoire must be expanded.

In this study we sought to develop a novel strategy to facilitate the detection of potential mucosal adjuvant compounds from a privileged-structure inspired chemical library based on their ability to activate MCs. MCs have recently been described to play a role in the development of adaptive immunity in several pro-inflammatory scenarios. Importantly, targeted activation of MCs using the small molecule MC activator c48/80 was found to induce robust systemic and mucosal immune responses in mice without any harmful side effects. Therefore, we reasoned that MCs might be used to identify novel agents that might be effective adjuvant compounds in vivo. The MC based screen is a high throughput strategy that has the capacity to screen thousands of chemical compounds from virtually any source. The high reproducibility of the assay based on internal plate controls that evaluate total β -hex, baseline β -hex release and a positive control using the MC activator compound 48/80 ensures that results from plate to plate can be compared and ranked based on MC degranulating activity as we described herein.

Others have used a semi-high-throughput screening assay³⁷ to identify possible adjuvant compounds based on induction of interleukin 8 (IL-8) by bovine epithelial cells. Screening a natural compound library containing 280 compounds revealed 3 compounds that consistently induced IL-8 production by bovine intestinal epithelial cells. Our mast cell degranulation screening assay also utilizes live cells but has an important difference when compared to the epithelial cell screening assay used by others³⁷. The epithelial screening assay depends upon the incubation of the cells with the test compound for 24 hours followed by testing of the culture supernatant for IL-8 produced in response to the test compound. Our mast cell degranulation is a rapid assay that tests the ability of the test compound to induce mast cell degranulation and release of granule contents into the tissue culture after a short incubation time of 30 minutes.

Evaluating novel compounds for cytotoxicity in cell-based screens is a common step in screening assays. However, we intentionally did not monitor cell viability due to the recent observations that alum, a US FDA approved vaccine adjuvant, involves cell death in its mechanism of adjuvant activity.³⁸ Alum has been suggested to be an inflammasome activator. The inflammasome is an intracellular multiprotein complex that is activated by a variety of microbial pathogens or alum that may result in "pyroptotic cell death".³⁹ Cell death via the pyroptotic pathway results in release of the cytosolic contents as well as interleukin 1 β (IL-1 β) secretion and both events are expected to be inflammatory.³⁹ While cytotoxicity is typically viewed as an adverse event and an outcome that should be avoided, the recent observation that alum provides effective and safe vaccine adjuvant activity via a mechanism that requires cell death³⁸ suggests that controlled cell death may be an effective strategy to mimic infection that ultimately provides safe and effective adjuvant activity. For this reason, cell viability was not measured since cell death may be an indicator of inflammasome activation and also an indicator of effective adjuvant activity. Mast cells have recently been reported to produce IL-1 β via an inflammasome-dependent mechanism.⁴⁰ Therefore, additional studies beyond the scope of this screening assay will be required to

determine if the mast cell activating compounds identified in this screen 1) provide effective adjuvant activity in vivo and 2) activate the inflammasome to provide IL-1 β production and/or pyroptotic cell death.

Cumulatively, in this screen, we evaluated 580 compounds from a benzopyran-based chemical library and successfully identified 12 compounds that could induce at least 5% MC/9 degranulation. Based on their structures and functionalities, these compounds fall into three distinct categories namely the benzylamine derivatives, the cyanostilbenes and the chalcones. Although comparative analysis mindful of c48/80 (Figure 8) revealed several essential structural features within each of the categories, a definitive structure-activity-relationship conclusion cannot be reached at this juncture. This finding may suggest these compounds mediate MC activation by engaging different receptors along the relevant biochemical pathway, and the structural diversity presented by the three class of compounds opens up new avenues for further molecular interrogations in the search for small molecule(s) with mucosal adjuvant activity.

Materials and Methods

Reagents

All chemicals were purchased from SIGMA ALDRICH (St. Louis, MO). All cell culture reagents were purchased from Invitrogen unless otherwise noted. The chemical library of 650 compounds was prepared with an adaptation of reported literature procedures¹⁶⁻¹⁸. All compounds used in biological testing were greater than 95% purity by ¹H NMR analysis.

Cell culture

The MC/9 mast cell line was purchased from ATCC (<http://www.atcc.org/>) and cultured according to their guidelines. All cells were cultured at 37 °C in a humidified water-jacketed incubator under 5% CO₂/95% air atmosphere.

β -hexosaminidase assays

MC/9 cells were used in the MC degranulation as described by others^{26,28} except that the assay was performed in 96-well V bottom plates at a density of 5×10^5 cells per well in 100 μ l sterile Tyrode's buffer. C48/80 (positive control) or library compounds were added to each well in a 100 μ l volume to the indicated final concentrations. Each sample was run in duplicate. Controls included MC/9 cells mixed with 0.2% Triton-X100 to measure total β -hex release, and samples mixed with Tyrode's buffer (vehicle control) as a measure of background β -hex release. After 30 minutes incubation with controls or test samples, each well is assayed for the presence of β -hex by combining 30 μ l of supernatant from each well with 10 μ l of substrate solution (p-nitrophenyl-N-acetyl- β -D-glucosaminide (NAG; SIGMA Cat. #N9376). Following a 30 minute incubation of 37 degrees C, a color change was induced by the addition of 100 μ l carbonate buffer per well followed by reading the absorbance at 405nm. Percent (%) degranulation was calculated using the following equation: $(\text{Experimental } \beta\text{-hex release} - \text{vehicle control } \beta\text{-hex release}) / (\text{Triton-X-100 } \beta\text{-hex release} - \text{vehicle control } \beta\text{-hex release}) \times 100$. Quality control criteria outlined in the text were used to grade the performance of each plate. Plates that did not meet these criteria were discarded and the assay was repeated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by awards from The NIH U01-AI082107; R21- DA029731; R01 DK077159. The authors wish to thank A*STAR (Agency for Science, Technology and Research), Singapore, for financial support.

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	1	2	3	4	5	6	7	8	9	10	11	12
A	Background	C48/80										
B	Background	C48/80										
C	Background	C48/80										
D	Background	C48/80										
E	Triton X-100	C48/80										
F	Triton X-100	C48/80										
G	Triton X-100	C48/80										
H	Triton X-100	C48/80										

Figure 1.

MC screening assay plate layout. Internal plate controls will include MC/9 cells treated with Tyrode's buffer alone (background), a maximum value control treated with the detergent Triton-X 100 (Triton-X 100), and MC/9 cells treated with the known MC activator c48/80. Each compound was evaluated in duplicate. Percent (%) degranulation will be calculated using the following equation: Percent (%) degranulation = (Experimental β -hex release – vehicle control β -hex release) / (Triton-X-100 β -hex release – vehicle control β -hex release) x 100. Compounds that elicit 5% degranulation of MC/9 cells will be selected for further evaluation.

Figure 2.
General synthetic scheme for the construction of benzopyran library.

Figure 3.

The MC activation screen for novel adjuvants is a consistent high throughput assay. **A.** Background β -hex release values for all 17 plates evaluated in this assay. Note that all values fall below the quality control criteria of 0.2. Maximal β -hex release values from MC/9 cells treated with the detergent Triton-X 100 from all 17 plates evaluated in the assay. Note that all values are greater than the quality control criteria of 1.0. **B.** Average percent β -hex release from MC/9 cells treated with the MC activator c48/80 (doses of 49.6, 198.3 and 793.5 μ M corresponding to 31.25, 125 and 500 μ g/ml) from all 17 plates evaluated in the assay. The individual values represent the calculated % degranulation from each assay plate and demonstrate that the assay performed reproducibly since the calculated % degranulation for each plate was within 3 standard deviations of the mean for each concentration of c48/80 tested.

Figure 4.
Notable structural classes of the benzopyran library found to elicit degranulation of MC/9 cells.

Figure 5.
Structure-activity-relationship of the benzylamine subset of the benzopyran library
(compound identification number over % MC/9 degranulation).

Figure 6. Structure-activity-relationship of the cyanostilbene subset of the benzopyran library (compound identification number over % MC/9 degranulation).

Figure 7.
Structure-activity-relationship of the chalcone subset of the benzopyran library (compound identification number over % MC/9 degranulation).

Figure 8.
Chemical structure of compound 48/80.

Table 1

Rank summary of the top 50 active chemical compounds evaluated in the MC degranulation assay.

Rank	Compound Number	% Degranulation
1	10	10.1
2	293	8.4
3	360	7.8
4	272	7.4
5	43	7.4
6	90	7.3
7	254	6.7
8	309	6.5
9	479	6.4
10	290	5.7
11	379	5.6
12	37	5.5
13	133	4.9
14	433	4.9
15	278	4.7
16	376	4.6
17	328	4.3
18	340	4.2
19	38	4.2
20	427	4.0
21	113	3.9
22	380	3.8
23	356	3.8
24	11	3.8
25	326	3.7
26	117	3.7
27	471	3.6
28	428	3.3
29	327	3.2
30	511	3.2
31	260	3.1
32	177	3.1
33	280	3.1
34	348	3.0
35	289	3.0
36	16	3.0

Rank	Compound Number	% Degranulation
37	302	2.9
38	242	2.9
39	459	2.9
40	319	2.8
41	358	2.8
42	369	2.8
43	95	2.7
44	324	2.7
45	337	2.7
46	291	2.7
47	274	2.6
48	89	2.6
49	152	2.6
50	245	2.6

Note that only 12 compounds were able to induce greater than 5.0% MC/9 degranulation. These compounds are highlighted in the supplementary sample submission list.