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High concentration electrophysiology-based fragment screen: Discovery of novel acid-sensing ion channel 3 (ASIC3) inhibitors

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ABSTRACT

The Merck Fragment Library was screened versus acid-sensing ion channel 3 (ASIC3), a novel target for the treatment of pain. Fragment hits were optimized using two strategies, and potency was improved from 0.7 mM to 3 μM with retention of good ligand efficiency and incorporation of reasonable physical properties, off-target profile, and rat pharmacokinetics.

Fragment screening represents a novel means to identify chemical leads for drug discovery projects.[1] This alternative to high-throughput screening (HTS) is based on the notion that sampling the chemical space of compounds within the molecular weight range 150–250 Da ('fragment space') is more efficient than sampling the chemical space represented by compounds of molecular weight 350+ (typical HTS space).[2] The efficiency gain is a direct result of the exponential size increase in chemical space which accompanies increases in molecular weight. The paradigm also benefits from an increased likelihood of finding efficient binding hits since smaller compounds have fewer opportunities for unfavorable interactions within the binding pocket.[2]

While potentially useful across drug target classes, fragment screening is particularly attractive for targets for which an HTS is not feasible. One obstacle to HTS screening is the lack of an assay with ultra high throughput; in this case, lead discovery teams are confined to using a lower throughput assay to screen subsets of corporate screening collections (10^3–10^4 compounds) instead of the entire collection (10^6 compounds). Since fragment screening enables an efficient sampling of chemical space via screening of a reduced number of compounds, it is a good fit for targets in this class.

Sodium ion channels are a class of drug target for which HTS assay development is challenging. FLIPR and VIPR approaches have been applied with limited success. These techniques depend on fluorescent dyes to indirectly measure channel activity. They frequently give high false positive rates due to channel independent depolarization by compound, compound fluorescence, and poor dye sensitivity. Attempts to mitigate some of these issues using sodium selective dyes have not improved fidelity significantly. For example, our internal effort using a FLIPR-based HTS screen against the acid-sensing ion channel 3 (ASIC3) produced few useful leads.[3,4] Many of the positive hits in the FLIPR HTS screen were inactive when followed-up using patch clamp electrophysiology. The few hits that were confirmed in patch clamp were not selective for ASIC3 and inhibited a wide variety of ion channels, including other ASIC isoforms. This result is consistent with observations from the ASIC3 literature, where lead discovery has been very challenging. Only computational approaches to lead finding are reported (no successful HTS has been published), and just three chemical series have been described: amiloride derivatives (which are non-selective),[3] A-317567[5] and analogues,[6] and aryl amidines.[4]

After eliminating the many false positive hits from our HTS effort, alternative lead sources were pursued. During the course of our screening effort, a medium-throughput automated patch clamp (PatchXpress, (PX); Molecular Devices, Inc., Sunnyvale, CA) assay was developed to support lead discovery and optimization. It was recognized that while the capacity of this assay was relatively low (80 data points/day) it was sufficient to enable a screen of the 1280-membered Merck Fragment Library[7] and this was ultimately carried out.

At the outset of this work, we were unaware of any precedence for fragment screening of an ion channel at high compound

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concentration (e.g., 1 mM) using an electrophysiology-based assay. Although the capacity of the PX assay appeared suitable for screening the Fragment Library, several challenges were evident: (1) due to the high concentrations of test compound required, the PX assay must tolerate relatively high concentrations of DMSO; (2) biochemical/functional assays have been shown inferior to binding assays for detection of low affinity fragment hits;[9] (3) generally, success in optimizing fragment hits to high potency leads depends on the availability of high resolution structural data (X-ray and/or NMR).[10] Despite these challenges, and in the absence of leads from any other source, we pursued this strategy.

The screening strategy is presented in Figure 1.[11] Initial hits were selected based on exhibiting >50% inhibition at 1 mM, and 56 compounds (4.4%) met this criterion. It is noteworthy that the hit rate in this screen for binders of an ion channel is similar to that obtained at Merck for fragments screens of other target classes (enzymes, protein–protein interactions, GPCRs). These hits were titrated (5-point) and prioritized based on ligand efficiency (LE). This parameter was calculated as described for ligand binding efficiency[12] with PX IC_{50} substituting for binding K_d ($LE = -RTln(IC_{50})/\#$ heavy atoms). Titrated hits with LE >0.3 were selected for further study (32 compounds, LE range 0.3–0.62, IC_{50} range 10–2050 μM).

These 32 compounds fell into structurally defined classes, and 12 distinct fragment series were delineated (Fig. 2).[13] Most hits contain basic groups, with 2-aminopyridine and benzylamine being the most common; all hits contain aromatic groups.

Optimization of LE, without increasing molecular weight, was achieved through similarity searching of the Merck sample collection and commercial vendors. The cap on molecular weight was put in place to bias toward identification of the optimal, highest LE *fragment* prior to any increase in MW, consistent with successful fragment optimizations disclosed previously.[14] It is worth noting that similarity searching of available fragment analogues, with no wet chemistry, was sufficient for the majority of hits. While the number of similars for a given hit was highly variable (<5 to >500), it was straightforward to select for representation of all the fragment hits, and the only constraint on analogue screening was PX assay throughput (250 fragment analogues were screened).

In general, similars were less potent than the initial fragment hits (two of many examples are shown in Fig. 2A). However, in some cases, potency and accompanying LE improvements were observed (Fig. 2B). Aside from the beneficial effect of a benzylic amine, there

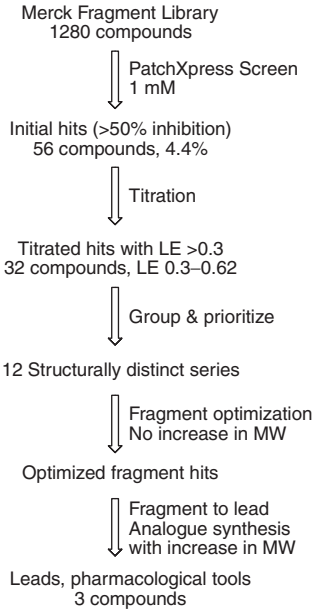


Figure 1. Electrophysiology-based fragment screening strategy for ASIC3.

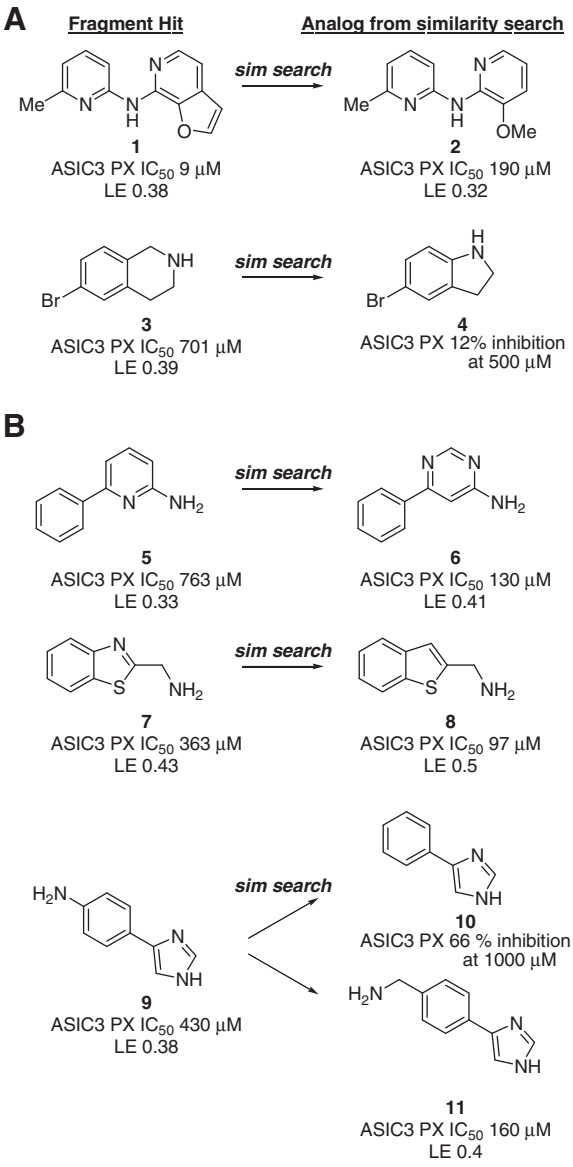
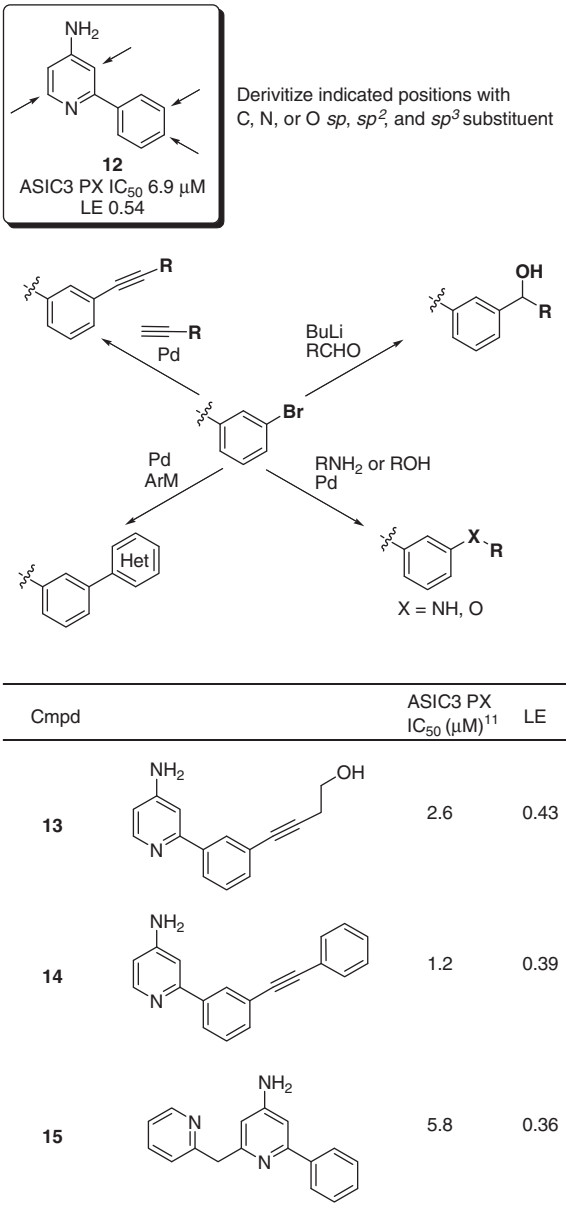


Figure 2. Fragment hits and optimization via similarity searching.

was no apparent general trend in which changes improved potency. For instance, reduction in basicity improves potency in the case of **5** to **6**, but the opposite trend holds for **9** to **10** to **11**.

Having maximized LE via similarity searching, the effort now turned to improving potency via synthesized analogues, and the cap on molecular weight was lifted. In the absence of structural information, the fragment optimization via growth was entirely empirical, and fragment linking was not considered. Two strategies were employed in growing the fragments: (1) a systematic plan with incorporation of a small group of substituents at every substitutable carbon of the fragment, or (2) an iterative analogue library approach in which synthetic handles on the fragment itself were exploited in parallel synthesis.

Fragment **12** exemplifies the systematic approach which is based on three principles (Fig. 3). First, without structural information the position of the fragment hit from which vacant pockets on the channel can be accessed is unknown; therefore growth from every substitutable position would comprehensively scan for such pockets. Second, the optimal geometry to access such a pocket is unknown, so to sample various geometries sp^3, sp^2, and *sp*-hybridized atoms would be incorporated at the position of attachment to the fragment hit. Third, a recent retrospective analysis of successful fragment



Cmpd		ASIC3 PX IC_{50} (μM)[11]	LE
13		2.6	0.43
14		1.2	0.39
15		5.8	0.36

Figure 3. Fragment optimization via systematic introduction of sp^3, sp^2, *sp*-hybridized substituents.

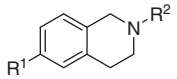
optimizations showed that an increase of 1 log unit in binding affinity can be expected for every 64 mass units added to the fragment;[10] in an attempt to use this finding as a prospective guide, substituents added were approximately 64 mass units and were judged by how close to a 10-fold gain in potency they achieved.

Execution of this strategy involved selection of 10–20 monomers per hybridization per position with sp^3 hybridization represented with C-, N-, and O-linkage to the fragment hit (250–500 analogues total). In practice, the plan was partially executed, with synthesis of 220 analogues at the indicated positions.[15] As shown in Figure 3, SAR for the series was generally flat, almost all analogues were inactive, and no substituent boosted potency by 10-fold; only decreases in LE versus fragment hit **12** were observed. Potency improvement with retention of acceptable LE was possible with addition of a phenyl 3-substituent (see **13** and **14**); in only one case (**15**) did installation of a substituent at a different position lead to an active compound; all other analogues with substitution on pyridine were inactive.

Optimization of fragments **1** and **3** proceeded via an iterative analogue library approach. Tetrahydroisoquinoline **3** was derivitized at nitrogen via parallel amide formation, sulfonamide formation, urea formation, and reductive alkylation, and at the isoquinoline 6-position via cross-coupling; in total 475 analogues were prepared (Table 1). Key SAR findings include the observation that N-substitution is poorly tolerated with the exception of **16** and **17**, and 5-methoxyindolyl-2-methyl is the most potency enhancing—a 70-fold improvement with slight loss in LE versus **3**. Substitution with aromatic groups at the tetrahydroisoquinoline 6-position was well tolerated, with a variety of both polar heterocycles and lipophilic aromatics providing 100–700× potency improvement with retention of good LE (**18–20**, **22–24**). Substitution at the same position with saturated ring systems eliminated activity. Attempts to combine potency enhancing features resulted in **21** and **24**, and **24** represents the optimal combination of potency, LE, and off-target properties achieved in this series (see Table 3). The most potent inhibitors in this series resemble the 7-substituted tetrahydroisoquinoline ASIC3 antagonist A-317567 disclosed by researchers at Abbott.[5]

Fragment **1** represents a larger and more potent starting point, and analogues demonstrated that pyridine 5- or 6-substitution was required and that replacement of the furopyridine was tolerated (Table 2). Installation of polar groups was possible, but gains in

Table 1
Optimization of the N2 and C6 substituents in the tetrahydroisoquinoline series



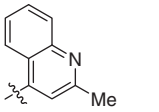
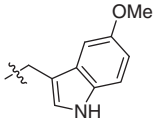
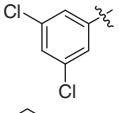
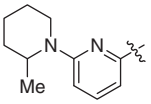
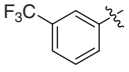
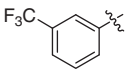
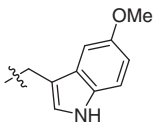
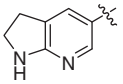
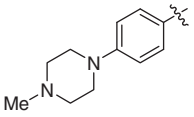
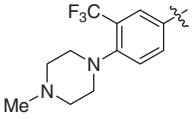
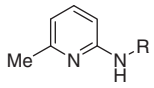
Compd	R^1	R^2	ASIC3 PX IC_{50} (μM)[11]	LE
3	Br	H	701	0.39
16	Br		27.6	0.28
17	Br		10.9	0.3
18		H	3.3	0.42
19		H	1.7	0.34
20		H	7.1	0.35
21			103	0.17
22		H	19.4	0.34
23		H	14.4	0.29
24		H	3.1	0.28

Table 2
Optimization of the 2-aminopyridine series



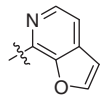
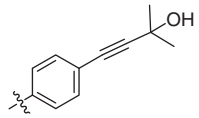
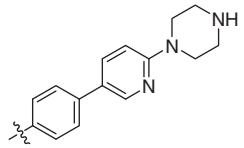
Compd	R	ASIC3 PX IC_{50} (μM)[11]	LE
1		9.0	0.38
25		17	0.33
26		3.8	0.29

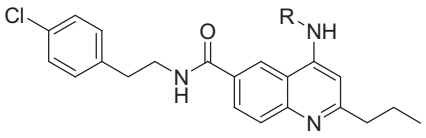
Table 3
Summary of data for **24** and **26**

	24	**26**
ASIC3 PX IC_{50} (μM)	3.1	3.8
$Ca_v1.2$ IC_{50} (μM)	>30	20
hERG bind K_i (μM)	nd	0.7
log *D* (HPLC)	2.13	1.99
PPB (human, rat)	89, 98.9%	90.2, 99.3%
CYP450 (3A4, 2C9, 2D6)	<35% inhibition at 10 μM	<45% inhibition at 10 μM
Rat pharmacokinetics:		
Cl (mL/min/kg)		11
V_d (L/kg)		3.5
$t_{1/2}$ (h)		4.8

potency required significant increases in molecular weight with an accompanying decrease in LE. A balanced analogue is **26**, which exhibits a reasonable balance among potency, LE, off-target selectivity, and good rat pharmacokinetics (see Table 3).

Compounds **24** and **26** represent potentially useful tools for the study of ASIC3 pharmacology; however, due to their high rat plasma protein binding they were not studied in an in vivo pain model. Nevertheless, based on their pharmacokinetics and off-target profiles they are reasonable leads for further optimization.

In summary, lead discovery for ASIC3 is a challenging problem. We pursued novel leads via an unprecedented high concentration electrophysiology-based assay of the Merck Fragment Library. This screen was successfully executed, provided a hit rate comparable to fragment screens run using more conventional binding and biochemical assays versus other targets, and detected high LE inhibitors in structural series not previously disclosed as ASIC3 blockers. Like other lead series which lack a basic amidine or guanidine group, achieving potency <100 nM proved challenging in these series. This may be a property of the channel—if there is a common binding site for these inhibitors, high potency may require high basicity—or it may reflect the difficulty inherent in optimizing any fragment hit in the absence of structural information. The present results are consistent with the analysis that advancing fragment hits to high potency (<100 nM) and/or to a preclinical development milestone in the absence of structural information is unlikely.[10] The disclosure of X-ray crystal structures for an inactive form of chicken ASIC1[16] (unavailable at the time this work was carried out) suggests that a structure-guided optimization of ASIC inhibitors may be possible. Regardless, it remains that ASIC3 is a challenging target for which the discovery of small molecule leads requires the full range of available techniques.



R = H, ASIC3 PX IC_{50} 9.6 μM

R = CH_3, ASIC3 PX 3% inhibition at 10 μM

Acknowledgments

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11. The potency of individual fragments was determined by their ability to block acid-evoked current in a mammalian expression system. Human embryonic kidney (HEK293) cells, stably expressing the human ASIC3 acid-sensing ion channel, were grown to 70–90% confluence in T75 cell culture flasks. Immediately prior to use, cells were treated with a 50/50 mixture of Trypsin-EDTA (GIBCO #25300) and Dulbecco's PBS (divalent-free; GIBCO #14190) solution for 2 min at 37 °C. Cells were gently triturated to achieve a suspension of ~6 million cells/mL and placed on the PX instrument. Cells were recorded at room temperature (25–29 °C) and held at a membrane potential of −60 mV. Cells were recorded in the 'whole cell' configuration with a defined intracellular and extracellular solution. Intracellular solution contained (in mM) 120 CsF, 30 CsCl, 2 $MgCl_2$, 10 EGTA, 5 HEPES, 5 NaF, and was adjusted to pH 7.3 with CsOH. Bath solution contained (in mM) 142 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 20 HEPES, 12 dextrose, and was adjusted to pH 7.6 using NaOH. For the pH 5.5 acid-activation bath solution, 20 mM MES was substituted for the HEPES buffer and pH adjusted to pH 5.5 with NaOH.
ASIC3 currents were elicited by a brief (3 s) application of pH 5.5 external solution. Solution exchange times were estimated to be <100 ms for wash-on and <250 ms for wash-off. Up to 16 cell recordings were obtained per run, whereby either a single concentration or a 5-pt titration of test compound was applied 2 min prior to and concurrent with pH 5.5 challenge. Baseline currents and recovery from inhibition assessment was achieved in these protocols with vehicle (1% DMSO) substituted for test compound. Percent inhibition was calculated by comparing peak ASIC3 current in test compound to the corresponding average of 2–3 baseline currents. IC_{50} values were determined by fitting 5-pt titration data to the Hill equation. Up to 2% DMSO could be applied as vehicle with no effect on current magnitude or kinetics. Control compounds (e.g., amiloride) demonstrated similar potency on the PX instrument as we obtained by manual patch clamp electrophysiology.
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