Probe Report

Title: ML302, a Novel Beta-lactamase (BLA) Inhibitor

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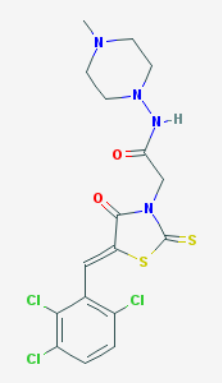
Abstract

VIM-2 and IMP-1 are Ambler class B metallo-β-lactamases (MBL) capable of hydrolyzing a broad-spectrum of β-lactam antibiotics. Although the discovery and development of MBL inhibitors continue to be an area of active research, an array of potent, non-selective (broad-acting) small molecule inhibitors is yet to be fully characterized. Here we describe a novel dual VIM-2/ IMP-1 inhibitor that exhibits efficacy in multiple clinical isolates. We therefore claim CID 53362017/SID 134220672 as a potent, broad acting VIM-2/IMP-1 probe (ML302). This compound was discovered from a medicinal chemistry effort that sought to improve the potency and efficacy of high-throughput screening (HTS) hits. During these chemistry efforts, we identified a rhodanine scaffold that exhibited activity against recombinant VIM-2 and IMP-1 in nitrocefin-based enzyme activity assays. Various secondary assays were run to determine its dual potency (VIM-2 IC_{50} = 548 nM; IMP-1 IC_{50} = 3.02 μM) and class B selectivity (it was inactive in TEM-1 and AmpC beta-lactamase enzymatic assays). Kinetic analyses demonstrated that ML302 behaves as a mixed mode uncompetitive/non-competitive inhibitor, with submicromolar K_i values against VIM-2 and IMP-1 (K_i = 183 ± 24 nM and 930 ± 97 nM, respectively). This represents an improvement in both our prior probe ML121 and the previously existing art compound Mitoxantrone, each of which inhibited only VIM-2. Subsequent studies revealed that this probe potentiates the activity of imipenem antibiotic in inhibiting growth of laboratory *E. coli* BL21 strains harboring VIM-2 and IMP-1, as well as clinical isolates YMC07 (VIM-2-containing *Acinetobacter* sp.), BAA-2146 (NDM-1-containing *Klebsiella pneumonia*), PA641 (VIM-2-containing *Pseudomonas aeruginosa*), and KN20 (IMP-1-containing *Pseudomonas aeruginosa*). This probe will serve as a valuable tool to elucidate the role of VIM-2 and IMP-1 in nosocomial beta-lactam antibiotic resistance.

Probe Structure and Characteristics:

DUAL VIM-2/IMP-1 Inhibitor Probe ML302
CID 53362017/ SID 134220672
MLS003940491 (SR-030000025555)



CID/ ML#	Target Name	IC50 (nM) [SID, AID]	Anti-target Name	IC_{50} (μM) [SID, AID]	Fold Selective	Secondary Assays: IC50 (nM) [SID, AID]
CID 53362017/ ML302	**VIM-2**	**Enzyme Assays:** IC_{50} = 548 nM [SID 125264855, AID 624079 K_i = 183 nM [SID134220672, AID 624083]	**IMP-1**	**Enzyme Assays:** IC_{50} = 3.018 μM [SID 125264855, AID 624085] K_i = 930 nM [SID134220672, AID 624084]	>5.5	**Enzyme counterscreens:** **TEM-1** IC_{50} >60 μM [SID125264855, AID 624092] **AmpC** IC_{50} >60 μM [SID125264855, AID 624090] **Imipenem Synergy assays:** *measured as a reduction (potentiation) of the MIC of imipenem in the following bacterial strains:* **YMC07/8/B3323 (VIM-2)** Achieves 32X Potentiation at 781 nM [SID 125264855, AID 624097] **BL21 (VIM-2)** Achieves 8X Potentiation at 3.125 μM [SID 125264855, AID 624081] **PA641 (VIM-2)** Achieves 4X Potentiation at 50 μM [SID 125264855, AID 624080] **BAA-2146 (NDM-1)** Achieves 2X Potentiation at >50 μM [SID 125264855, AID 624082] **KN20 (IMP-1)** Achieves 2X Potentiation at >50 μM [SID 125264855, AID 624095] **BL21 (IMP-1)** Achieves 4X Potentiation at 781 nM [SID 125264855, AID 624097]

Recommendations for scientific use of the probe

Limitations in state of the art. We previously submitted a probe report describing a selective VIM-2 inhibitor (ML121). This probe was potent and active against the VIM-2 target. However, it lacked activity against the IMP-1 enzyme, which might limit its usefulness in clinically resistant bacterial strains that may express more than one β-lactamase. Another non-competitive VIM-2 inhibitor, mitoxantrone (1,4-dihydroxy-5,8-bis([2-([2-hydroxyethyl]amino)ethyl]amino)-9,10-anthracenedione; CID 4212), is a type II topoisomerase inhibitor that disrupts DNA synthesis and DNA repair in both healthy cells and cancer cells [1]. Like ML121, its inhibition is also selective to VIM-2 and its intense color limits Mitoxantrone's use in certain assay detection formats. Another prior art compound, 4-chloromercuribenzoic acid (*p*CMB; CID 1730), is a slowly reversible/irreversible VIM-2 inhibitor shown to have a synergistic effect with β-lactam antibacterials in VIM-2-expressing bacteria.[2] Unfortunately, this cysteine-reactive reagent is known to have several off-target activities, lessening its value for mechanistic studies.

Probe Applications. Here we demonstrate that our probe ML302 (CID 53362017/ SID 134220672/ SR-03000002555) blocks the enzymatic activities of VIM-2 and IMP-1 in biochemical assays, with no apparent activity against class A (TEM-1) and class C (AmpC) β-lactamases. When dosed in clinical isolates, ML302 shows no toxicity; when co-dosed with imipenem, ML302 significantly increases imipenem's efficacy (MIC). These findings have significant implications for studies that probe the enzymology of VIM-2 and IMP-1, especially mechanistic studies to better understand these enzymes' broad-spectrum activity against various beta-lactam based antibiotics. Further, this probe can be useful for experiments that aim to inhibit VIM-2 and IMP-1 activity, without inhibiting TEM-1 or AmpC activity. In a broader role, the non-selectivity, potency, and efficacy of this compound will enable further investigations into the biological and biochemical roles of metallo-β-lactamase enzymes, and may be useful in the design of inhibitors to inhibit these clinically relevant enzymes and reduce the public health burden of antibiotic resistance.

Expected end-users of the probe in the research community. The probe can be used by researchers studying microbiology, antibiotic chemistry, β-lactamase enzymology. Thus, it is conceivable that scientists in diverse fields will be able to apply ML302 to elucidate the role of VIM-2 and IMP-1 in bacterial resistance pathways and investigate mechanisms of VIM-2 and IMP-1 inhibition in biochemical and microbiology-based assays.

Relevant biology of the probe. VIM-2 and IMP-1 are a zinc-dependent, Ambler Class "B" β-lactamases that hydrolyze β-lactam based antibiotics (e.g. penicillins, carbapenems), rendering them ineffective. No VIM-2 inhibitors yet exist for clinical use, and all VIM-2 inhibitors reported to date have been designed to bind zinc or modify cysteine found in the enzyme's active site. Further, no dual-acting inhibitors exist which block activity of both of these enzymes. Therefore, selective, non-competitive VIM-2 inhibitors are desired to probe VIM-2 function exclusive of active site inhibition.

1 Introduction

The emergence of Gram-negative bacteria that exhibit multi-drug resistance, combined with the lack of new antibiotics, poses a public health challenge [3]. The production of bacterial β-lactamase enzymes, in particular, is a common mechanism of drug resistance [4-6]. The β-lactamases evolved from bacteria with resistance to naturally-occurring β-lactams or penams [7], agents which inhibit the transpeptidase involved in cell wall biosynthesis [8]. Human medicine adapted these agents into synthetic antibiotics such as penicillins, cephalosporins, carbapenems, and monobactams that contain a 2-azetidone ring [7, 9]. The metallo-β-lactamases (MBL) are zinc-dependent class B β-lactamases that hydrolyze the β-lactam ring, rendering the

antibiotic ineffective [8, 10]. Increasingly, nosocomial beta-lactam antibiotic resistance arises in *P. aeruginosa*, *Enterobacteriaceae*, and other pathogenic bacteria via gene transfer of B1 MBLs [6, 11], including IMP (active on IMiPenem) [12] and VIM (Verona IMipenemase) [13, 14]. For two of these enzymes, VIM-2 and IMP-1, no inhibitors exist for clinical use [8, 11]. Thus, the identification of MBL inhibitors would provide useful tools for reducing nosocomial infections and elucidating their mechanism of action [2, 15]. In the present report, we identify and characterize a compound belonging to a novel rhodanine scaffold that inhibits both VIM-2 and IMP-1 with submicromolar K_i values. This represents a significant advance in the field of broad acting β-lactamase inhibitors.

2.1 Assays

Table 1. *Assays from the HTS campaign and prior probe discovery effort (ML121):*

AID	Assay Name	Assay Type	Target	Powder Sample
1527	Primary biochemical HTS assay to identify inhibitors of VIM-2.	Primary Assay (1X%INH)	VIM-2	No
1556	Epi-absorbance primary biochemical HTS assay to identify inhibitors of IMP-1 metallo-beta-lactamase.	Primary Assay (1X%INH)	IMP-1	No
1856	Epi-absorbance-based counterscreen for selective VIM-2 inhibitors: biochemical HTS assay to identify inhibitors of IMP-1 metallo-beta-lactamase.	Counterscreen (3X%INH)	IMP-1	No
1857	FRET-based counterscreen assay for selective VIM-2 inhibitors: biochemical HTS assay to identify epi-absorbance assay artifacts.	Counterscreen (3X%INH)	VIM-2 (CCF2)	No
1860	Epi-absorbance-based confirmation biochemical HTS assay to identify selective inhibitors of VIM-2 metallo-beta-lactamase.	Confirmation (3X %INH)	VIM-2	No
1866	Epi-absorbance-based counterscreen assay for selective VIM-2 inhibitors: biochemical HTS assay to identify inhibitors of TEM-1 serine-beta-lactamase.	Counterscreen (3X%INH)	TEM-1	No
1919	Epi-absorbance-based dose response biochemical high throughput screening assay for selective inhibitors of VIM-2 metallo-beta-lactamase.	Dose Response (3X IC50)	VIM-2	No
1920	Epi-absorbance-based counterscreen for selective VIM-2 inhibitors: dose response biochemical high throughput screening assay to identify inhibitors of IMP-1 metallo-beta-lactamase.	Dose Response Counterscreen (3X IC50)	IMP-1	No
1925	Epi-absorbance-based counterscreen for selective VIM-2 inhibitors: dose response biochemical high throughput screening assay to identify inhibitors of TEM-1 serine-beta-lactamase.	Dose Response Counterscreen (3X IC50)	TEM-1	No
1926	FRET-based counterscreen for selective VIM-2 inhibitors: dose response biochemical high throughput screening assay to identify epi-absorbance assay artifacts.	Dose Response Counterscreen (3X IC50)	VIM-2 (CCF2)	No
1927	FRET-based counterscreen for selective VIM-2 inhibitors: dose response biochemical high throughput screening assay to identify inhibitors of IMP-1 metallo-beta-lactamase.	Dose Response Counterscreen (3X IC50)	IMP-1 (CCF2)	No
1854	Summary of probe development efforts to identify selective inhibitors of VIM-2 metallo-beta-lactamase.	Summary	VIM-2	No
2128	Late stage results from the probe development efforts to identify inhibitors of VIM-2: probe results	Late Stage AID (probe)	VIM-2	Yes
2317	Late stage results from the probe development efforts to identify selective inhibitors of VIM-2 metallo-beta-lactamase: Prior art results.	Late Stage AID (prior art)	VIM-2	Yes
504620	Late stage Assay provider assay to determine imipenem Synergy of probe ML121	Late Stage AID (probe ML121)	VIM-2	Yes

Table 2. *Assays for the current probe discovery effort (ML302):*

AID	Assay Name	Assay Type	Target	Powder Sample
624079	Absorbance-based Biochemical Nitrocefin Substrate Hydrolysis Assays (IC50)	Dose Response (2X IC_{50}) (probe ML302)	VIM-2	Yes
624085			IMP-1	Yes
624092			TEM-1	Yes
624090			AmpC	Yes
624083	Absorbance-based Biochemical Nitrocefin Substrate Hydrolysis Assays (Ki and Km)	Dose Response (2X)	VIM-2	Yes
624084			IMP-1	Yes
624096	Absorbance based Imipenem Antibiotic Synergy Assay (MIC4X): YMC07/B3323	Dose Response (2X)	VIM-2	Yes
624081	Absorbance based Imipenem Antibiotic Synergy Assay (MIC4X): BL21	Dose Response (2X)	VIM-2	Yes
624080	Absorbance based Imipenem Antibiotic Synergy Assay (MIC4X): PA641	Dose Response (2X)	VIM-2	Yes
624082	Absorbance based Imipenem Antibiotic Synergy Assay (MIC4X): BAA-2146	Dose Response (2X)	NDM-1	Yes
624095	Absorbance based Imipenem Antibiotic Synergy Assay (MIC4X): KN20	Dose Response (2X)	IMP-1	Yes
624097	Absorbance based Imipenem Antibiotic Synergy Assay (MIC4X): BL21	Dose Response (2X)	IMP-1	Yes

(Click on the hyperlinks to obtain itemized protocols directly from PubChem; also see Summary AID 1854)

VIM-2 Inhibition Assays (Epi-Absorbance-based; Nitrocefin) (PubChem AIDs: AID 1527, AID 1860, AID 1919, AID 2128, AID 2317, and *AID 624079).* The purpose of this assay is to identify compounds that act as inhibitors of the VIM-2 β-lactamase. This biochemical epi-absorbance-format assay employs the cephalosporin nitrocefin as the VIM-2 substrate, and takes advantage of the fluorescent properties of white microtiter plates [16]. Nitrocefin is a yellow chromogenic substrate (Imax = 395 nm) that is hydrolyzed by β-lactamases to yield a red product with increased absorbance properties (Imax = 495 nm) that quenches plate fluorescence by absorbing the plate's emission light [16]. In this assay, test compounds are incubated with purified VIM-2 enzyme and nitrocefin in detergent-containing buffer at room temperature. The reaction is stopped by the addition of EDTA, followed by measurement of well fluorescence. As designed, compounds that inhibit VIM-2 will inhibit nitrocefin hydrolysis, inhibit generation of red product, and inhibit quenching of plate fluorescence, resulting in an

increase in well fluorescence. Compounds were tested in singlicate (AID1527) and triplicate (AID 1860) at a final nominal concentration of 5.6 µM, in a 10-point 1:3 dilution series starting at a nominal concentration of 55.7 µM (AID 1919, *AID* 2128, *AID* 2317, and *AID* 624079).

IMP-1 Inhibition Counterscreens (Epi-absorbance-based; Nitrocefin) (AID 1556, *AID* 1856, *AID* 1920, *AID* 2128, *AID* 2317, and *AID* 624085). The purpose of this assay is to identify compounds that act as inhibitors of the IMP-1 β-lactamase. This biochemical epi-absorbance-format assay employs the cephalosporin nitrocefin as the IMP-1 substrate, and takes advantage of the fluorescent properties of white microtiter plates [16]. This assay also serves as a counterscreen to determine whether compounds identified as possible VIM-2 selective inhibitors are non-selective due to inhibition of IMP-1. Nitrocefin is a yellow chromogenic substrate (Imax = 395 nm) that is hydrolyzed by β-lactamases to yield a red product with increased absorbance properties (Imax = 495 nm) that quenches plate fluorescence by absorbing the plate's emission light [16]. In this assay, test compounds are incubated with purified IMP-1 enzyme and nitrocefin in detergent-containing buffer at room temperature. The reaction is stopped by the addition of EDTA, followed by measurement of well fluorescence. As designed, compounds that inhibit IMP-1 will inhibit nitrocefin hydrolysis, inhibit generation of red product, and inhibit quenching of plate fluorescence, resulting in an increase in well fluorescence. Compounds were tested in singlicate (AID 1556) or in triplicate (AID 1856) at a final nominal concentration of 5.6 µM, and in triplicate using a dilution series starting at a nominal test concentration of 60 micromolar (AID 1920, *AID* 2128, *AID* 2317, and *AID* 624085).

2.2 Probe Chemical Characterization

The synthesis of the probe and analytical characterization data are probe ML302 are provided in **Section 2.3**. ML302 was obtained with >98% purity according to ^{1}H NMR and LCMS analysis. However, ML302 had relatively poor solubility characteristics, so we also generated a more soluble HCl salt, identified as CID53384679. The HCl salt was also obtained with >96% purity by 1H NMR and LCMS analysis.

Solubility. The solubility of probe ML302 (synthesized and registered by the SRISMC as SR-03000002555-2/ SID 134220672/ CID 53362017) was measured in phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic and a pH of 7.4) at room temperature (23°C). The solubility of was found to be <1 µM. In an attempt to achieve improved solubility, a series of salts (including the HCl salt, CID 53384679) were synthesized and studied. However, salt HCl CID 53384679 also had poor solubility (<1 µM) in this standard pH 7.4 PBS buffer assay system. However, probe ML302 and HCl salt CID53384679 are fully soluble at 100 µM in 70:30 DMSO/water, and both have solubility >100 µM at pH = 3 aqueous solution. A sample of the HCl salt CID 53384679 is also soluble >100 µM in D_2O (^{1}H NMR measurements performed at this concentration). Both probe ML302 and the HCl salt CID 53384679 are fully soluble under the conditions of the VIM-2 biochemical assays and antibacterial assays described in this probe report.

Stability. The stability of probe ML302 was measured at room temperature (23ºC) in PBS (no antioxidants or other protectants; DMSO concentration below 0.1%). The stability, represented by the half-life, was found to be very poor. **Figure 1** provides graphs showing loss of compound with time over a 48 hour period with a minimum of 6 time points. The table at the end of this section indicates the percent of compound remaining at the end of the 48 hours. These data suggest that the probe ML302 and HCl salt CID 53384679 are highly unstable under these assay conditions. However, these data are not consistent with our experience with these compounds when handled under other conditions. Rather, we suspect that these data reflect solubility problems with the compound under the assay conditions. For example, HCl salt CID 53384679 proved to be fully stable, at 100 µM, in D_2O over a two-week monitoring period (^{1}H NMR study)—conditions under which it is fully soluble. Similarly, probe ML302 and HCl salt CID 53384679 were fully stable at 100 µM in 70/30 DMSO-D_2O over a two-week period, in presence of air (^{1}H NMR study)—conditions, again, under which they are fully soluble. ML302 and HCl salt CID 53384679 (both 1 µM) were fully stable (24 h monitoring) at pH 3 in PBS with 20% DMSO, and displayed >93% stability (over 24 h) at pH 7 PBS with 20% DMSO. The latter value

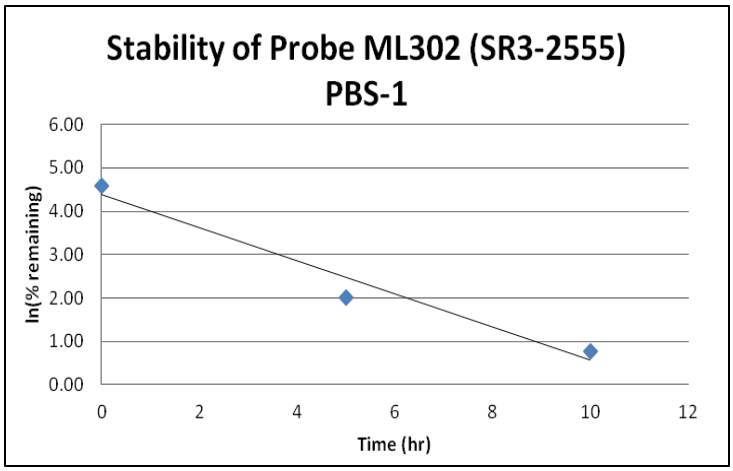
undoubtedly reflects the limit of solubility of these compounds under these conditions. Apparently, the HCl salt CID 53384679 is converted to the free base (ML302) at pH 7.4.

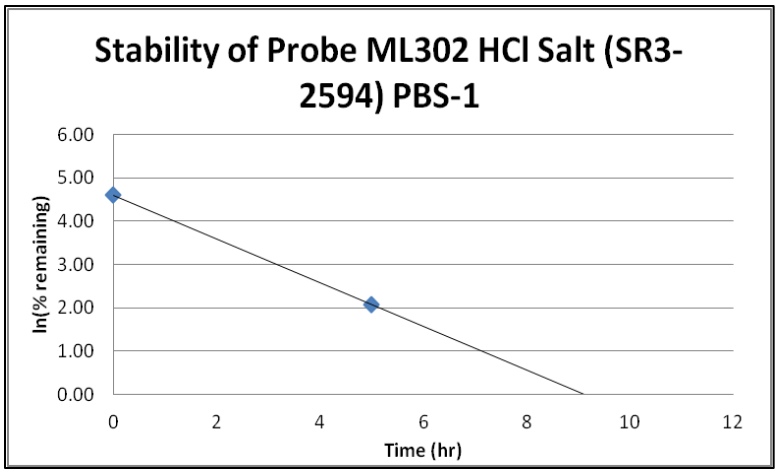
Probe ML302, and its HCl salt were measured for its ability to form glutathione adducts. At concentrations of 100 µM reduced GSH, 10 µM of the new probe does not appear to be a Michael acceptor [17, 18]. Evidently, the chlorine substituents at the 2,6-position of the phenyl ring hinder the double bond such that these compounds are not highly active as Michael acceptors.

Table 3. *Solubility of ML302*

Compound	MW	SR Number	CID	SID	Solubility in PBS (µM)	Michael Acceptor 100 µM GSH trap	Stability in PBS t1/2 (hr)
New Probe ML302	479.8	SR-03000002555-2	53362017	134220672	<1 µM	No	Section 2.2
Probe ML302 HCl salt	516.3	SR-03000002594-2	53384679	134220676 (synthesis)	<1 µM	No	Section 2.2

Figure 1*. Stability Analysis of ML302 and its HCl salt*

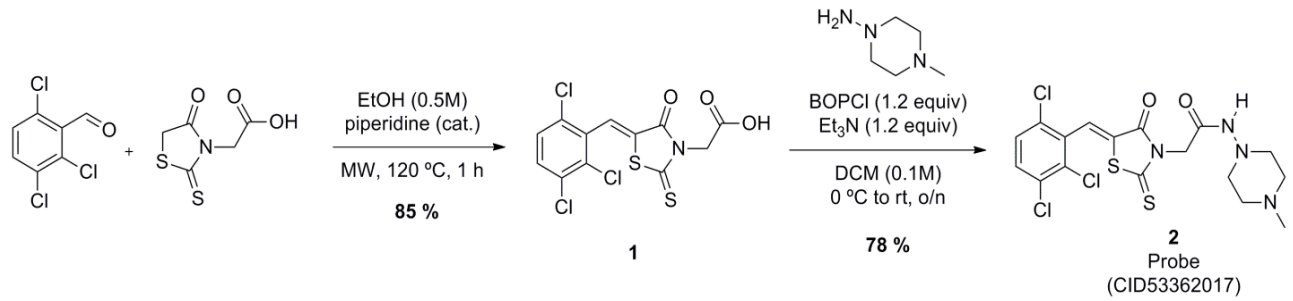




2.3 Probe Preparation

VIM-2 inhibitor probe ML302 was synthesized by a two-step procedure involving the condensation of 2,3,6-trichlorobenzaldehyde with rhodanine-3-acetic acid, followed by coupling of the product carboxylic acid **1** with 1-amino-4-methyl-piperazine [19].

Figure 2. *Synthesis of VIM-2 Inhibitor Probe CID 53362017 (ML302) (Compound 2)*



2,3,6-Trichlorobenzaldehdye (1.676 g, 8.0 mmol, 1 equiv, purchased from Oakwood Product, Inc. # 043332) and rhodanine-3-acetic acid (1.530 g, 8.0 mmol, 1 equiv, purchased from Alfa Aesar, # B22244-06) were weighed into a 20-mL microwave vial. Ethanol (16 mL, to give a reaction concentration of 0.5 M) was added followed by piperidine (4 drops). The vial was sealed and submitted to microwave irradiation at 120 °C for 1 h.

The solvent was then removed and the crude product was recrystallized from hot ethanol and water. The solid was filtered, rinsed with water and dried under high vacuum yielding compound **1** as a yellow solid (2.59 g, 85%)**: ^{1}H NMR** (400 MHz, *d6*-DMSO) δ 13.56 (1H, br s), 7.84 (1H, s), 7.87-7.84 (1H, m), 7.81 (1H, d, *J* = 8.8 Hz), 7.67 (1H, d, *J* = 8.7Hz), 4.70 (2H, s). **^{13}C NMR** (100 MHz, *d6*-DMSO) δ 192.6, 167.2, 164.9, 133.0, 132.2, 131.5, 131.4, 131.2, 131.1, 129.8, 128.6, 45.6.

A solution of **1** (76 mg, 0.2 mmol, 1 equiv.) and BOPCl (61 mg, 0.24 mmol, 1.2 equiv) in dichloromethane (2 mL, to give a reaction concentration of 0.1 M) in a 5-mL round-bottomed flask was cooled to 0 °C using an ice/water bath under an inert atmosphere. Freshly distilled triethylamine (35 µL, 0.24 mmol, 1.2 equiv) was added and the solution was allowed to stir at 0 °C for 30 min. The cold bath was removed and 1-amino-4-methyl-piperazine (29 µL, 0.24 mmol, 1.2 equiv, purchased from Acros Organics, # 251391000) was added. The mixture was allowed to warm to room temperature and was stirred overnight. The crude product, obtained by removal of all solvents volatile compounds in vacuo, was directly purified by flash chromatography (silica gel, dichloromethane-methanol 90/10, R_f = 0.29) yielding compound **2** as an inseparable mixture of *E*/*Z* isomers in a 1:3 ratio (75 mg, 78 %, yellow solid, mp = 184-186 °C) (**Figure 2**). The chemical purity of **2** is >98% according to HLPC analysis.

Data for VIM-2 Inhibitor <u>Probe, CID 53362017 (Compound 2), *Z* isomer (major)</u>: ^{1}H NMR (400 MHz, *d6*-DMSO) δ 9.08 (1H, s), 7.83 (1H, s), 7.82 (1H, d, *J* = 8.7 Hz), 7.68 (1H, d, *J* = 8.8 Hz), 4.91 (2H, s), 2.99-2.62 (6H, m), 2.43-2.02 (2H, m), 2.17 (3H, s); **^{13}C NMR** (100 MHz, *d6*-DMSO) δ 192.8, 166.0, 165.1, 133.0, 132.2, 131.5, 131.4, 131.3, 131.2, 129.8, 128.2, 55.1, 54.3, 45.3, 45.2. **MS** ([M+H]+): 481.1 (100%), 479.4 (71%), 483.0 (39%). **IR** (cm^{-1}): 3049, 2927, 2808, 1731, 1687, 1627, 1402, 1366, 1323, 1286, 1273, 1193, 1175, 1051, 1008, 816, 725.

Partial Data for (minor) E isomer of VIM-2 Inhibitor Probe <u>CID 53362017</u>: ^{1}H NMR (400 MHz, *d6*-DMSO) δ 9.37 (1H, s), 7.82 (1H, d, *J* = 8.7 Hz), 7.81 (1H, s), 7.68 (1H, d, *J* = 8.8 Hz), 4.56 (2H, s), 2.99-2.62 (6H, m), 2.43-2.02 (2H, m), 2.15 (3H, s).

All anti-bacterial and enzyme assays performed with the VIM-2 inhibitor probe, CID 53362017 (compound 2) were performed with the 3:1 mixture of olefin isomers. The two olefin isomers of the Probe were not separated, since prior studies with three probe analogs (SR-6818, SR-2448 and SR-2450) (**Figure 3**) indicated that the separated olefin isomers rapidly re-isomerized to the original 3:1 mixtures. (The olefin isomers of SR-6818, SR-2448 and SR-2450 were separated by preparative HPLC. In all three cases, the separated isomers had converted back to the original 3:1 mixtures in the time required to concentrate the HPLC fractions prior to NMR analysis).

Figure 3. *Probe analogs*

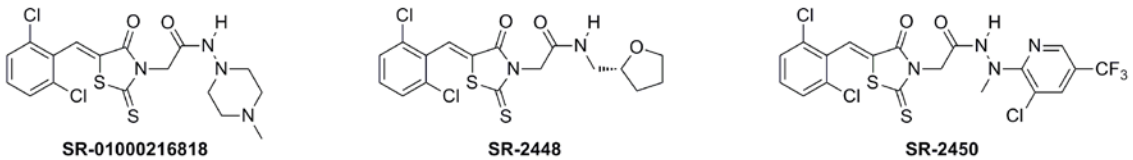
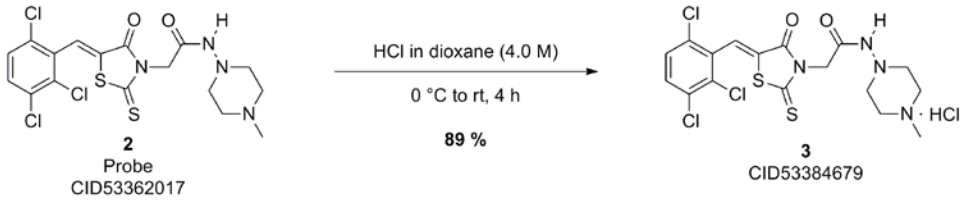


Figure 4. *Synthesis of Hydrochloride Salt of the Probe (CID 53384679, Compound 3)*



A solution of hydrogen chloride in dioxane (4.0M, 4.1 mL, 16.4 mmol, 80 equiv) was slowly added at 0 °C to compound **2** (100 mg, 0.21 mmol, 1 equiv) under an inert atmosphere. The cold bath was removed and the mixture was allowed to stir at room temperature for 4 h. The resulting heterogeneous solution was filtered and the yellow precipitate **3** (**Figure 4**) was thoroughly washed with diethyl ether and dried under vacuum. No further purification was performed and the hydrochloride salt was obtained as a mixture of *E*/*Z* isomers in a 1:1 ratio (96 mg, 89 %, yellow solid, mp = 203-206 °C).

Data for VIM-2 Inhibitor CID 53384679 (Compound 3), *Z* isomer: ^{1}H NMR (400 MHz, *d6*-DMSO) δ 11.21 (1H, br s), 9.41 (1H, s), 7.82 (1H, s), 7.81 (1H, d, *J* = 8.5 Hz), 7.67 (1H, d, *J* = 8.5 Hz), 4.99 (2H, s), 3.49-3.00 (8H, m), 2.73 (3H, s); **^{13}C NMR** (100 MHz, *d6*-DMSO) δ 192.8, 165.1, 162.1, 132.9, 132.2, 131.5, 131.4, 131.2, 131.1, 129.8, 128.3, 52.5, 51.8, 50.7, 45.3. **MS** ([M+H]+): 481.1 (100%), 479.5 (74%), 483.0 (38%). **IR** (cm^{-1}): 2936, 2464, 1724, 1692, 1564, 1437, 1335, 1190, 1176, 1100, 1055, 984, 898, 814.

Partial Data for E isomer of VIM-2 Inhibitor Compound 3 CID 53384679: ^{1}H NMR (400 MHz, *d6*-DMSO) δ 11.21 (1H, br s), 9.97 (1H, s), 7.81 (1H, d, *J* = 8.5 Hz), 7.80 (1H, s), 7.67 (1H, d, *J* = 8.5 Hz), 4.60 (2H, s), 3.49-3.00 (8H, m), 2.71 (3H, s).

3 Results

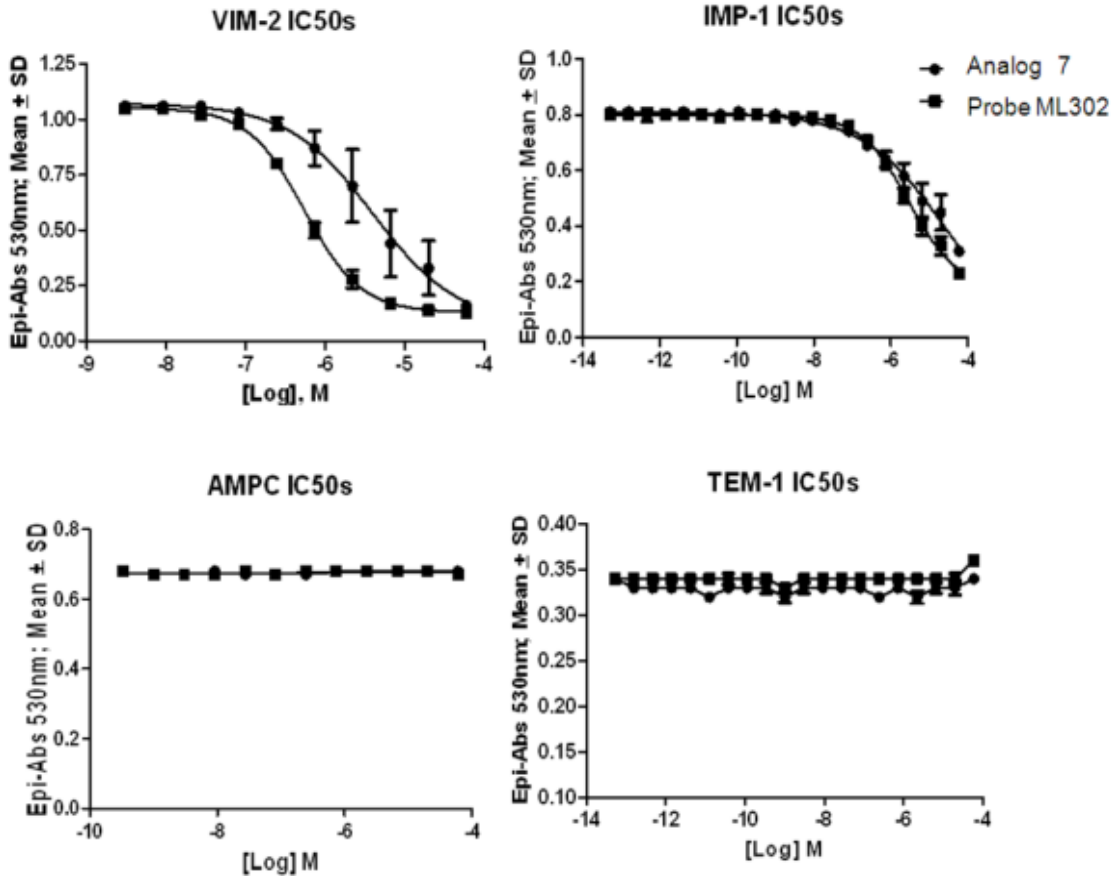
3.1 Summary of Screening Results

During the medicinal chemistry effort following our initial VIM-2 selective inhibitor project, the rhodanine scaffold, exemplified by SR-01000216818/CID1733268, exhibited activity against the anti-target enzyme, IMP-1. This suggested that this scaffold could be a source of broader-acting dual inhibitors of bacterial MBLs which might serve as tools for novel antibiotic discovery. In order to assess the efficacy of the new scaffold, we tested powder samples of leads in a series of biochemical and bacterial-based assays. Initial studies with recombinant VIM-2 protein revealed that several compounds in this scaffold exhibited potencies less than 5 micromolar. *The broad acting MBL probe (ML302) was identified from this scaffold.*

3.2 Dose Response Curves for Probes

Dose response curves for probe ML302 are shown in **Figure 5** against all enzymes tested. The probe shows selective potency to the VIM-2 and IMP-1 enzymes. For comparison, SR-01000216818/CID 1733268 ("Analog 7") is also graphed. Potency values are reported in the cover page of this report.

Figure 5. *Dose response curves for ML302*



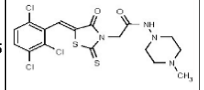
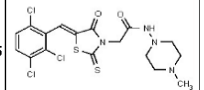
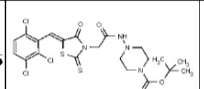
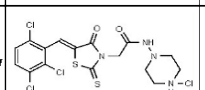
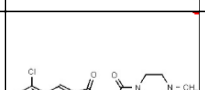
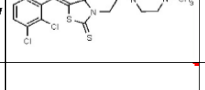
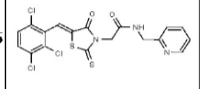
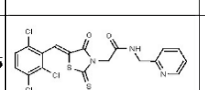
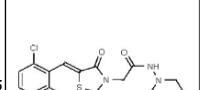
3.3 Scaffold/Moiety Chemical Liabilities

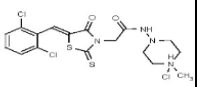
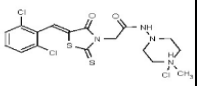
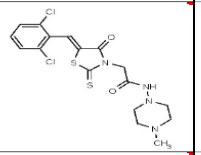
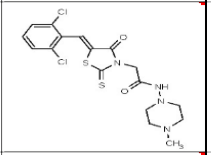
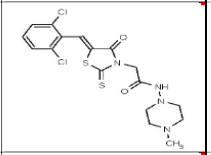
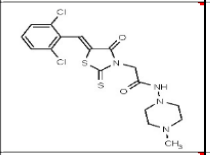
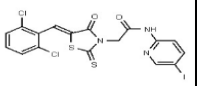
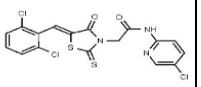
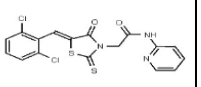
There is no known instability or chemical liabilities associated with the chemical scaffold of probe ML302. The compounds appear to be fully stable during all routine handling operations (synthesis chromatographic purification of the probe, NMR studies, storage as powders, etc.). Issues with solubility of the probe and its HCl salt are discussed in a previous section. The only issue we have observed is that the probe and its HCl salt are unstable at pH 10, due to amide hydrolysis at this basic pH. Therefore, handling of probe ML302 at pH > 7.4 is not recommended.

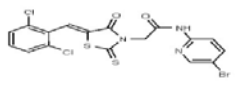
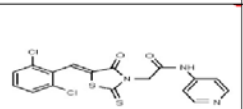
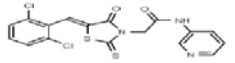
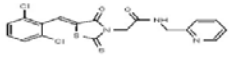
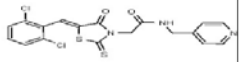
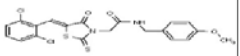
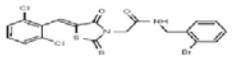
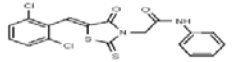
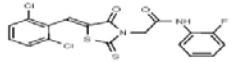
3.4 SAR Tables

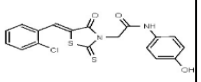
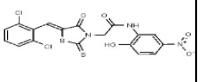
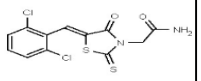
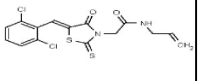
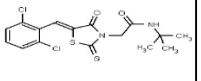
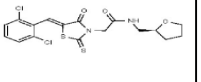
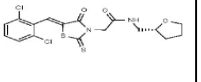
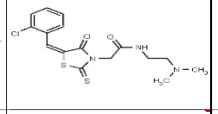
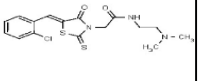
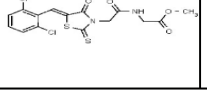
Describe SAR & chemistry strategy (including structure and data) that led to the probe. Following submission of our initial probe report describing a *selective* VIM-2 inhibitor probe (ML121), compounds in the rhodanine scaffold appeared to have activity for both VIM-2 and IMP-1, suggesting a broad-acting MBL inhibitor probe could be developed. Powder and re-synthesized samples of selected compounds were tested by the assay provider in several biochemical and bacterial assays to determine potency, efficacy, and selectivity. These efforts resulted in probe ML302 and related analogs. The synthesis of ML302, presented in Section 2.3 of this probe report, is amenable to synthesis of additional analogs by substituting different aldehydes instead of 2,3,6-trichlorobenzaldehyde using the condensation of rhodanine-3-acetic acid; use of different amines instead of 1-amino-4-methyl-piperazine in the coupling with carboxylic acid **1**. As summarized in the following SAR tables, more than 90 such analogs have been synthesized and tested. Preliminary efforts indicate that introduction of amino acid side chains in the rhodanine scaffold also lead to active compounds. Additional studies of such analogs will be performed in the future.

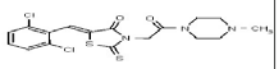
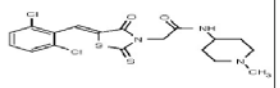
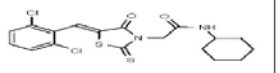
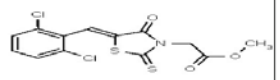
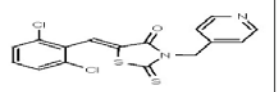
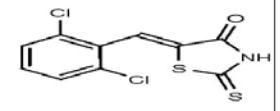
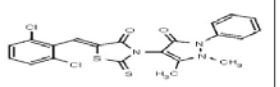
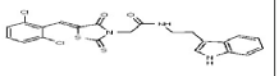
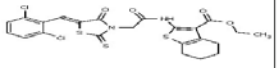
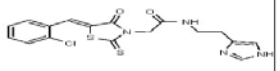
Metallo-beta Lactamase Inhibitor SAR Table: Rhodanine Scaffold

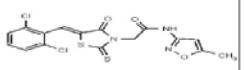
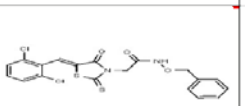
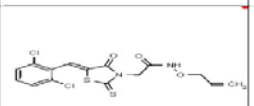
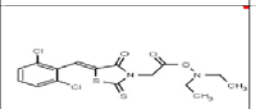
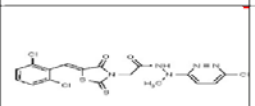
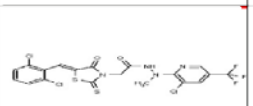
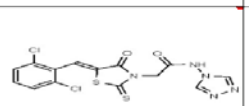
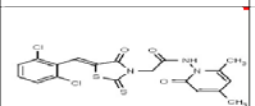
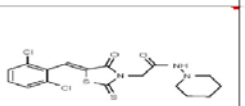
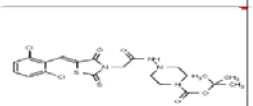
Compound Information								Bioassays												Biochemical Assays							
								BL21 (VIM-2)		YMC07/B3323 (VIM-		BAA-2146 (NDM-1)		PA641 (VIM-2)		KN20 (IMP-1)		BL21 (IMP-1)		VIM-2	IMP-1	TEM-1	AmpC	VIM-2 Ki		IMP-1 Ki Assay	
Compound	SR-Number	Structure	CID	SID	MLS	Vendor	Vendor Catalog ID	MIC 4x (µM)	Imipenem Potentiation	MIC 4x (µM)	Imipenem Potentiation	MIC 4x (µM)	Imipenem Potentiation	MIC 4x (µM)	Imipenem Potentiation	MIC 4x (µM)	Imipenem Potentiation	MIC 4x (µM)	Imipenem Potentiation	IC50 (uM)	IC50 (uM)	IC50 (uM)	IC50 (uM)	Ki (nM)	Km (uM)	Ki (nM)	Km (uM)
Probe	SR-030000002555-1		53362017	125264855		TSRI	None	3.125	8X	0.781	32X	>50	2X	50	4X	>50	2X	0.781		0.5481	3.018	>60	>60	ND	ND	ND	ND
Probe ML302 (Sample to NIH)	SR-030000002555-2		53362017	134220672	003940491	TSRI	None	ND		0.391	32X	ND		ND		ND		ND		ND	ND	ND	ND	183 +/- 24	14.8 +/- 3 Mixed mode inhibitor	930 +/- 97	6.4 +/- 1.3 Mixed mode inhibitor
Analog 1	SR-030000002674-1		53495083	126723255	003940492	TSRI	None	1.563		0.781	16X	>50		>50		>50		3.125		ND	ND	ND	ND	ND	ND	ND	ND
Analog 2	SR-030000002704-1		54579799	131269026	003940493	TSRI	None	12.5		0.781	32X	>50	2X	>50	2X	>50		25		ND	ND	ND	ND	ND	ND	ND	ND
Analog 3	SR-030000002705-1		54579797	131269027		TSRI	None	6.25		1.563	16X	>50		50	4X	>50		0.781	8X	ND	ND	ND	ND	ND	ND	ND	ND
Analog 3	SR-030000002705-2		54579797	134220673	003940494	TSRI	None	ND		0.781	32X	ND		ND		ND		ND		ND	ND	ND	ND	ND	ND	ND	ND
Analog 4	SR-030000002672-1		53495082	126723253		TSRI	None	3.125		1.563	16X	>50		>50		>50		0.781		ND	ND	ND	ND	ND	ND	ND	ND
Analog 4	SR-030000002672-2		53495082	134220674	003940495	TSRI	None	ND		1.563	32X	ND		ND		ND		ND		ND	ND	ND	ND	ND	ND	ND	ND
Analog 5	SR-030000002594-1		53384679	125311278		TSRI	None	3.125	8X	1.563	16X	>50		>50	Achievs 2X	>50		1.563		ND	ND	ND	ND	ND	ND	ND	ND
Analog 5	SR-030000002594-2		53384679	134220676	003940496	TSRI	None	ND		0.781	16X	ND		ND		ND		ND		ND	ND	ND	ND	ND	ND	ND	ND

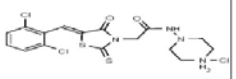
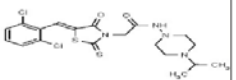
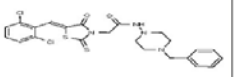
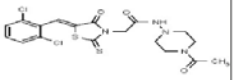
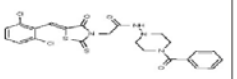
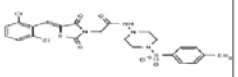
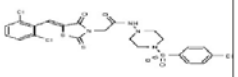
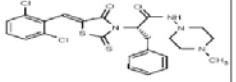
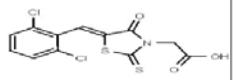
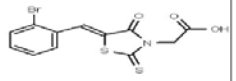
Compound Information								Bioassays										Biochemical Assays		
								BL21 (VIM-2)		YMC07/B3323 (VIM-2)		BAA-2146 (NDM-1)		PA641 (VIM-2)		KN20	BL21	VIM-2	IMP-1	VIM-2 Ki
Compound	SR-Number	Structure	CID	SID	MLS	Vendor	Vendor Catalog ID	MIC 4x (μM)	Imipenem Potentiation	MIC 4x (μM)	Imipenem Potentiation	MIC 4x (μM)	Imipenem Potentiation	MIC 4x (μM)	Imipenem Potentiation	MIC 4x (μM)	MIC 4x (μM)	IC50 (nM)	IC50 (nM)	Ki (nM)
Analog 6	SR-03000002441-1		53308605	124767346		TSRI	None	12.5	8X	3.125	32X	>100	2X	>100	2X	>100	3.125	ND	ND	ND
Analog 6 (resynthesized for submission to NIH)	SR-03000002441-2		53308605	134220675	003940497	TSRI	None	ND		3.125	32X	ND		ND		ND	ND	ND	ND	ND
Analog 7 (Purchased)	SR-01000216818-4		1733268	1356318091		ChemBridge	5680564											4.55		1.781 +/- 0.384
Analog 7 (resynthesized)	SR-01000216818-6		1733268	123083180		TSRI	None	ND		25	Prepared in 75/25 DMSO/H2O	ND		>100		ND	ND	ND	ND	ND
Analog 7 (purchased for reference)	SR-01000216818-7		1733268	124360376		ChemBridge	5680564	ND		3.125	Prepared in neat DMSO	ND		ND		ND	ND	ND	ND	ND
Analog 7 (resynthesized)	SR-01000216818-8		1733268	124384965	003940498	TSRI	None	6.25	8X	6.25	32X	>100		>100	2X	>100	25	3.98	31.9	ND
Analog 8	SR-03000002375-1		53257035	124398381		TSRI	None	>100		ND		ND		ND		ND	ND	ND	ND	ND
Analog 9	SR-03000002376-1		53257030	124398382		TSRI	None	>100		ND		ND		ND		ND	ND	ND	ND	ND
Analog 10	SR-03000002377-1		53257026	124398383		TSRI	None	>100		ND		ND		ND		ND	ND	ND	ND	ND

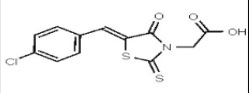
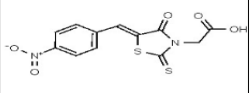
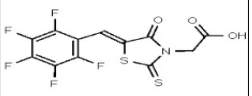
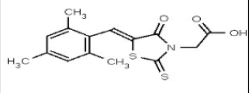
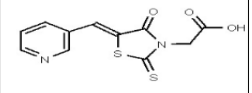
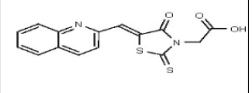
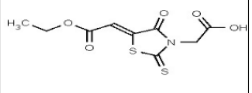
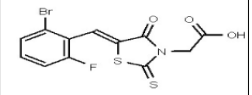
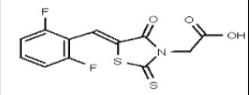
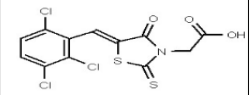
Compound Information								Bioassays									
								BL21 (VIM-2)		YMC07/B3323 (VIM-2)		BAA-2146 (NDM-1)		PA641 (VIM-2)		KN20	BL21
Compound	SR-Number	Structure	CID	SID	MLS	Vendor	Vendor Catalog ID	MIC 4x (µM)	Imipenem Potentiation	MIC 4x (µM)	Imipenem Potentiation	MIC 4x (µM)	Imipenem Potentiation	MIC 4x (µM)	Imipenem Potentiation	MIC 4x (µM)	MIC 4x (µM)
Analog 11	SR-03000002380-1		53257031	124398386		TSRI	None	>100		ND		ND		ND		ND	ND
Analog 12	SR-03000002381-1		53257037	124398404		TSRI	None	12.5	8X	ND		ND		ND		ND	ND
Analog 13	SR-03000002461-1		1382782	125001880		Vitas-M Lab	STL035211	ND		6.25	16X	ND		ND		ND	ND
Analog 14	SR-03000002378-1		53257011	124398384		TSRI	None	6.2	8X	6.25	16X	>100		>100	2X	>100	ND
Analog 15	SR-03000002379-1		53257036	124398385		TSRI	None	12.5		6.25	16X	ND		ND		ND	ND
Analog 16	SR-03000002395-1		53301882	124756530		TSRI	None	>100		ND		ND		ND		ND	ND
Analog 17	SR-03000002396-1		53301881	124756531		TSRI	None	>100		ND		ND		ND		ND	ND
Analog 18	SR-03000002433-1		53308614	124767337		TSRI	None	>100		ND		ND		ND		ND	ND
Analog 19	SR-03000002434-1		53308615	124767338		TSRI	None	>100		ND		ND		ND		ND	ND

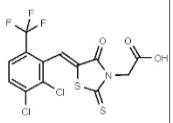
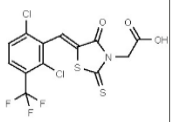
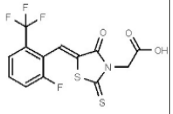
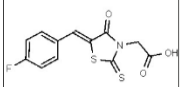
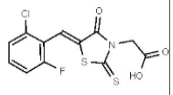
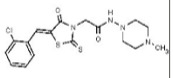
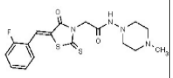
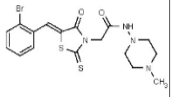
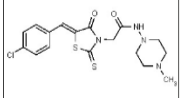
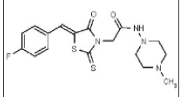
Compound Information								Bacterial Assays (Strain and MBL)										Biochemical Assays		
								BL21 (VIM-2)		YMC07/B3323 (VIM-2)		BAA-2146 (NDM-1)		PA641 (VIM-2)		KN20	BL21	VIM-2	IMP-1	VIM-2 Ki
Compound	SR-Number	Structure	CID	SID	MLS	Vendor	Vendor Catalog ID	MIC 4x (μM)	Inipocon Potentiation	MIC 4x (μM)	Inipocon Potentiation	MIC 4x (μM)	Inipocon Potentiation	MIC 4x (μM)	Inipocon Potentiation	MIC 4x (μM)	MIC 4x (μM)	IC50 (nM)	IC50 (nM)	Ki (nM)
Analog 20	SR-03000002455-1		2049116	125001873		Innovapharm Ltd.	STT-00175051	>100		ND		ND		ND		ND	ND	ND	ND	ND
Analog 21	SR-03000002460-1		5724963	125001879		Vitas-M Lab	STL035210	3.13		50		ND		ND		ND	ND	ND	ND	ND
Analog 22	SR-03000002385-1		53301880	124756519		TSRI	None	25		ND		ND		ND		ND	ND	ND	ND	ND
Analog 23	SR-03000002386-1		53301896	124756520		TSRI	None	>100		ND		ND		ND		ND	ND	ND	ND	ND
Analog 24	SR-03000002398-1		53301889	124756533		TSRI	None	>100		ND		ND		ND		ND	ND	ND	ND	ND
Analog 25	SR-03000002447-1		53313357	124807342		TSRI	None	>100		ND		ND		ND		ND	ND	ND	ND	ND
Analog 26	SR-03000002448-1		53313358	124807343		TSRI	None	>100		ND		ND		ND		ND	ND	ND	ND	ND
Analog 27	SR-03000002457-1		17592954	125001875		Vitas-M Lab	STK626562	100		ND		ND		ND		ND	ND	ND	ND	ND
Analog 28	SR-03000002459-1		50739720	125001877		Innovapharm Ltd.	STT-00362432	100		ND		ND		ND		ND	ND	ND	ND	ND
Analog 29	SR-00000009750-1		53308608	124767339		TSRI	None	>100		ND		ND		ND		ND	ND	ND	ND	ND

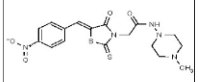
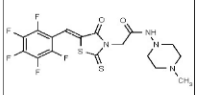
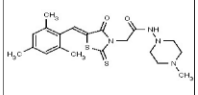
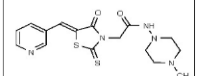
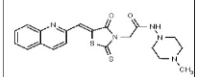
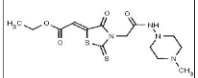
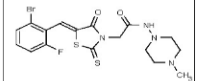
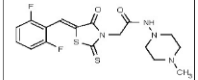
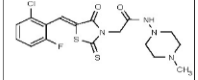
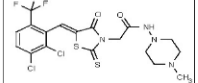
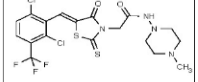
Compound Information								Bacterial Assays (Strain and MBL)			
								BL21 (VIM-2)		YMC07/B3323 (VIM-2)	
Compound	SR-Number	Structure	CID	SID	MLS	Vendor	Vendor Catalog ID	MIC 4x (μM)	Imipenem Potentiation	MIC 4x (μM)	Imipenem Potentiation
Analog 30	SR-03000002382-1		53257017	124398405		TSRI	None	12.5	8X	6.25	16X
Analog 31	SR-03000002383-1		53301893	124756517		TSRI	None	12.5	8X	ND	
Analog 32	SR-03000002432-1		53308616	124767336		TSRI	None	>100		ND	
Analog 33	SR-03000002374-1		1560158	124384967		TSRI	None	12.5	8X	ND	
Analog 34	SR-03000002431-1		53308606	124767335		TSRI	None	12.5	8X	ND	
Analog 35	SR-03000002442-1		1280940	124767347		TSRI	None	100		ND	
Analog 36	SR-03000002458-1		1908865	125001876		Vitas-M Lab	STK861771	12.5	8X	ND	
Analog 37	SR-03000002397-1		53301874	124756532		TSRI	None	>100		ND	
Analog 38	SR-03000002454-1		1554607	125001872		Innovapharm Ltd.	STT-00172281	>100		ND	
Analog 39	SR-03000002456-1		6226188	125001874		Vitas-M Lab	STK601034	>100		ND	

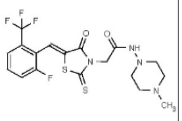
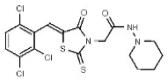
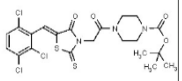
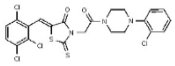
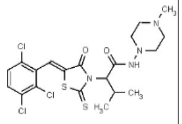
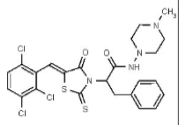
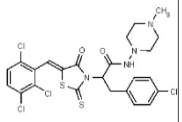
Compound Information								Bacterial Assays (Strain and MBL)								
								BL21 (VIM 2)		YMC07/B3323 (VIM 2)		BAA 2146 (NDM 1)		PA641 (VIM 2)		KN20
Compound	SR-Number	Structure	CID	SID	MLS	Vendor	Vendor Catalog ID	MIC 4x (µM)	Imipenem Potentiation	MIC 4x (µM)	Imipenem Potentiation	MIC 4x (µM)	Imipenem Potentiation	MIC 4x (µM)	Imipenem Potentiation	MIC 4x (µM)
Analog 40	SR-03000002462-1		5858470	125001881		Vitas-M Lab	STL035212	100		ND		ND		ND		ND
Analog 41	SR-03000002446-1		53313356	124807341		TSRI	None	>100		ND		ND		ND		ND
Analog 42	SR-03000002451-1		53313350	124807346		TSRI	None	>100		ND		ND		ND		ND
Analog 43	SR-03000002452-1		53338906	125001870		TSRI	None	6.2	8X	12.5	8X	>100		>100		>100
Analog 44	SR-03000002449-1		53313352	124807344		TSRI	None	>100		ND		ND		ND		ND
Analog 45	SR-03000002450-1		53313351	124807345		TSRI	None	>100		ND		ND		ND		ND
Analog 46	SR-03000002480-1		53346521	125011825		TSRI	None	100		ND		ND		ND		ND
Analog 47	SR-03000002481-1		53346520	125011826		TSRI	None	ND		1.563	32X	ND		ND		ND
Analog 48	SR-03000002384-1		53301887	124756518		TSRI	None	6.2	8X	3.125	16X	ND		>100		>100
Analog 49	SR-03000002453-1		53338912	125001871		TSRI	None	6.2	8X	3.125	16X	>100		>100		>100

Compound Information								Bacterial Assays (Strain and MBL)								
								BL21 (VIM 2)		YMC07/B3323 (VIM 2)		BAA 2146 (NDM 1)		PA641 (VIM 2)		KN20
Compound	SR Number	Structure	CID	SID	MLS	Vendor	Vendor Catalog ID	MIC 4x (µM)	Imipenem Potentiation	MIC 4x (µM)	Imipenem Potentiation	MIC 4x (µM)	Imipenem Potentiation	MIC 4x (µM)	Imipenem Potentiation	MIC 4x (µM)
Analog 50	SR-03000002482-1		53346529	125011827		TSRI	None	3.125	8X	3.125	16X	>50		>50	2X	>50
Analog 51	SR-03000002588-1		53384710	125311272		TSRI	None	12.5	16X	6.25	32X	>50		ND		>50
Analog 52	SR-03000002589-1		53384681	125311273		TSRI	None	ND		3.125		>50		>50		>50
Analog 53	SR-03000002590-1		53384726	125311274		TSRI	None	ND		6.25	32X	>50		>50	2X	>50
Analog 54	SR-03000002591-1		53384676	125311275		TSRI	None	ND		3.125	16X	>50		>50		>50
Analog 55	SR-03000002592-1		53384686	125311276		TSRI	None	ND		25		ND		ND		ND
Analog 56	SR-03000002593-1		53384728	125311277		TSRI	None	ND		>50	2X	ND		ND		ND
Analog 57	SR-03000002737-1		54669692	131465571		TSRI	None	ND		25	8X	ND		ND		ND
Analog 58	SR-03000002373-1		1201205	124384966		TSRI	None	3.125	8X	12.5	16X	>100		>100		>100
Analog 59	SR-03000002519-1		1205102	125258648		TSRI	None	ND		>50	2X	ND		ND		ND

Compound Information								Bacterial Assays (Strain and MBL)			
								BL21 (VIM-2)		YMC07/B3323 (VIM-2)	
Compound	SR-Number	Structure	CID	SID	MLS	Vendor	Vendor Catalog ID	MIC 4x (μM)	Imipenem Potentiation	MIC 4x (μM)	Imipenem Potentiation
Analog 60	SR-03000002520-1		1201114	125258649		TSRI	None	ND		>50	2X
Analog 61	SR-03000002521-1		1622325	125258651		TSRI	None	ND		>50	2X
Analog 62	SR-03000002522-1		13337884	125258652		TSRI	None	ND		>50	2X
Analog 63	SR-03000002523-1		1201949	125258653		TSRI	None	ND		>50	
Analog 64	SR-03000002524-1		1201311	125258654		TSRI	None	ND		>50	
Analog 65	SR-03000002525-1		5943467	125258655		TSRI	None	ND		>50	
Analog 66	SR-03000002526-1		53356622	125258656		TSRI	None	ND		>50	
Analog 67	SR-03000002527-1		53356627	125258657		TSRI	None	ND		>50	2X
Analog 68	SR-03000002528-1		53356652	125258658		TSRI	None	ND		>50	2X
Analog 69	SR-03000002529-1		53356632	125258660		TSRI	None	ND		6.25	16X

Compound Information								Bacterial Assays (Strain and MBL)								
								BL21 (VIM2)		YMC07/B3323 (VIM2)		BAA-2146 (NDM1)		PA641 (VIM2)		KN20
Compound	SR-Number	Structure	CID	SID	MLS	Vendor	Vendor Catalog ID	MIC 4x (μM)	Imipenem Potentiation	MIC 4x (μM)	Imipenem Potentiation	MIC 4x (μM)	Imipenem Potentiation	MIC 4x (μM)	Imipenem Potentiation	MIC 4x (μM)
Analog 70	SR-03000002530-1		53356630	125258661		TSRI	None	ND		6.25	16X	ND		ND		ND
Analog 71	SR-03000002531-1		53356624	125258662		TSRI	None	ND		6.25	8X	ND		ND		ND
Analog 72	SR-03000002586-1		53384688	125311270		TSRI	None	25	8X	25	2x	>50		ND		>50
Analog 73	SR-01000199849-2		1381767	125258650		TSRI	None	ND		>50		ND		ND		ND
Analog 74	SR-01000214156-2		1201186	125258659		TSRI	None	ND		>50	2X	ND		ND		ND
Analog 75	SR-01000686826-3		1594367	125001878		Vitas-M Lab	STK994030	50		ND		ND		ND		ND
Analog 76	SR-01000699784-2		1553748	125001882		Vitas-M Lab	STL035275	100		ND		ND		ND		ND
Analog 77	SR-03000002543-1		53361995	125264843		TSRI	None	ND		50	4X	ND		ND		ND
Analog 78	SR-03000002544-1		53361997	125264844		TSRI	None	ND		>50		ND		ND		ND
Analog 79	SR-03000002545-1		53362020	125264845		TSRI	None	ND		>50		ND		ND		ND

Compound Information								Bacterial Assays (Strain and MBL)								
								BL21 (VIM-2)		YMC07/B3323 (VIM-2)		BAA-2146 (NDM-1)		PA641 (VIM-2)		KN20 (IMP-1)
Compound	SR-Number	Structure	CID	SID	MLS	Vendor	Vendor Catalog ID	MIC 4x (µM)	Imipenem Potentiation	MIC 4x (µM)	Imipenem Potentiation	MIC 4x (µM)	Imipenem Potentiation	MIC 4x (µM)	Imipenem Potentiation	MIC 4x (µM)
Analog 80	SR-03000002546-1		53362005	125264846		TSRI	None	ND		>50		ND		ND		ND
Analog 81	SR-03000002547-1		53362003	125264847		TSRI	None	ND		50	2X	ND		ND		ND
Analog 82	SR-03000002548-1		53362016	125264848		TSRI	None	ND		25	4X	ND		ND		ND
Analog 83	SR-03000002549-1		53362008	125264849		TSRI	None	ND		50	4X	ND		ND		ND
Analog 84	SR-03000002550-1		53362009	125264850		TSRI	None	ND		>50		ND		ND		ND
Analog 85	SR-03000002551-1		53362004	125264851		TSRI	None	ND		50	2X	ND		ND		ND
Analog 86	SR-03000002552-1		53362006	125264852		TSRI	None	ND		50	2X	ND		ND		ND
Analog 87	SR-03000002553-1		53362014	125264853		TSRI	None	ND		25	4X	ND		ND		ND
Analog 88	SR-03000002554-1		53362019	125264854		TSRI	None	12.5		3.125	32X	>50	2X	>50		>50
Analog 89	SR-03000002556-1		53362002	125264856		TSRI	None	1.563	8X	3.125	32X	>50	2X	50	4X	>50
Analog 90	SR-03000002557-1		53362007	125264857		TSRI	None	0.781	8X	3.125	16X	>50	2X	>50	2X	>50

Compound Information								Bacterial Assays (Strain and MBL)								
Compound	SR-Number	Structure	CID	SID	MLS	Vendor	Vendor Catalog ID	BL21 (VIM-2)		YMC07/B3323 (VIM-2)		BAA-2146 (NDM-1)		PA641 (VIM-2)		KN20 (IMP-1)
								MIC 4x (μM)	Imipenem Potentiation	MIC 4x (μM)	Imipenem Potentiation	MIC 4x (μM)	Imipenem Potentiation	MIC 4x (μM)	Imipenem Potentiation	MIC 4x (μM)
Analog 91	SR-03000002587-1		53384696	125311271		TSRI	None	25	8X	3.125	2X	>50	2X	>50		>50
Analog 92	SR-03000002673-1		53495084	126723254		TSRI	None	ND		3.125	16X	>50		>50		>50
Analog 93	SR-03000002706-1		54579793	131269028		TSRI	None	ND		6.25	16X	ND		ND		ND
Analog 94	SR-03000002707-1		54579790	131269029		TSRI	None	ND		>50		ND		ND		ND
Analog 95	SR-03000002806-1		56596523	134228483		TSRI	None	ND		1.563	32X	ND		ND		ND
Analog 96	SR-03000002807-1		56596515	134228484		TSRI	None	ND		1.563	32X	ND		ND		ND
Analog 97	SR-03000002808-1		56596526	134228485		TSRI	None	ND		1.563	32X	ND		ND		ND

3.5 Cellular Assays

The probe was tested in a variety of bacterial assays performed by the assay provider to determine the probe's selectivity, efficacy and mechanism of action.

MIC and Imipenem Antibiotic Synergy Assays [AID 624081 BL21 (VIM2); AID 624097 BL21 (IMP1); AID 624096 YMC07 (VIM2); AID 624082 BAA2146 (NDM1); AID 624080 PA641 (VIM2); AID 624095 KN20 (IMP1)]. Using a checkerboard microdilution method, probe ML302 was tested for its ability to potentiate the efficacy of imipenem on VIM-2-expressing *E. coli* (BL21/VIM-2) [20-22]. The probe exhibited synergy with imipenem when present at concentrations as low as 3.13 µM, potentiating imipenem's activity 8-fold. Significantly, probe ML302 exhibited 32-fold imipenem potentiation in clinically relevant VIM-2-transformed *Acinetobacter* species YMC07/B3323: MIC range = 390-781 nM). Synergy was also observed by probe ML302 in other clinical isolates including *K. pneumonia* BAA-2146 expressing NDM-1 [2-fold], *P. aeruginosa* expressing VIM-2 (PA641) [4-fold], and *P. aeruginosa* expressing IMP-1 (KN20) [2-fold].

3.6 Profiling Assays

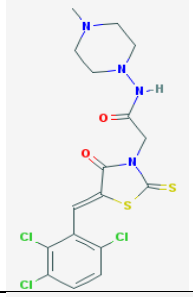
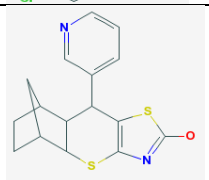
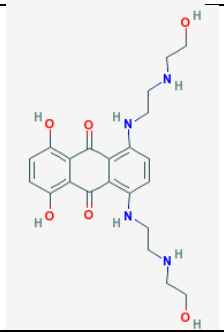
The identified VIM-2/IMP-1 inhibitor probe ML302 was tested in biochemical and bacterial MIC assays to determine inhibition of VIM-2, IMP-1, TEM-1, AmpC, as well as assess its ability to potentiate the antibacterial activity of imipenem. Based on the results of these assays, it was agreed by the SRIMSC and assay provider that additional profiling was not required at this time.

4 Discussion

4.1 Comparison to existing art and how the new probe is an improvement

Compared to our prior probe ML121 and prior art mitoxantrone, the new probe ML302 exhibits improved biochemical potency, antibacterial activity, and IMP-1 activity.

Table 4. *Comparison of ML302 to existing Art*

Name	Structure	SR Number	CID	SID	MLS ID	VIM-2 IC50	IMP-1 IC50	VIM-2 Ki	TEM-1 IC50	AmpC IC50	Synergy	PubChem Activity
Probe ML302		SR-0300000 2555-2	5336 2017	134220672	MLS-00394 0491	548 nM	3.02 µM	183 nM	>60 µM	>60 µM	3.125 µM (VIM-2) 0.781 µM (IMP-1) (SID 12526485 5)	None
Probe ML121		SR-0100077 5688-1	4870 494	24790728 (MLSMR); 85856282 (purch); 103911139 (synth)	MLS-00068 0027	223 nM	>60 µM	148 nM	>60 µM	ND	Active (12.5 µM) (TFA salt)	6/ 309 (1.9%)
Prior art Mitoxan trone		SR-0100007 6001-7	4212 (5458 171)	56424031 (85856281)	MLS 00133 3711	powder 0.63 µM	> 56 µM	1.5 µM (non-competi tive):	> 56 µM	ND	Not applicable	40/139 (28.8%)

4.2 Mechanism of Action Studies

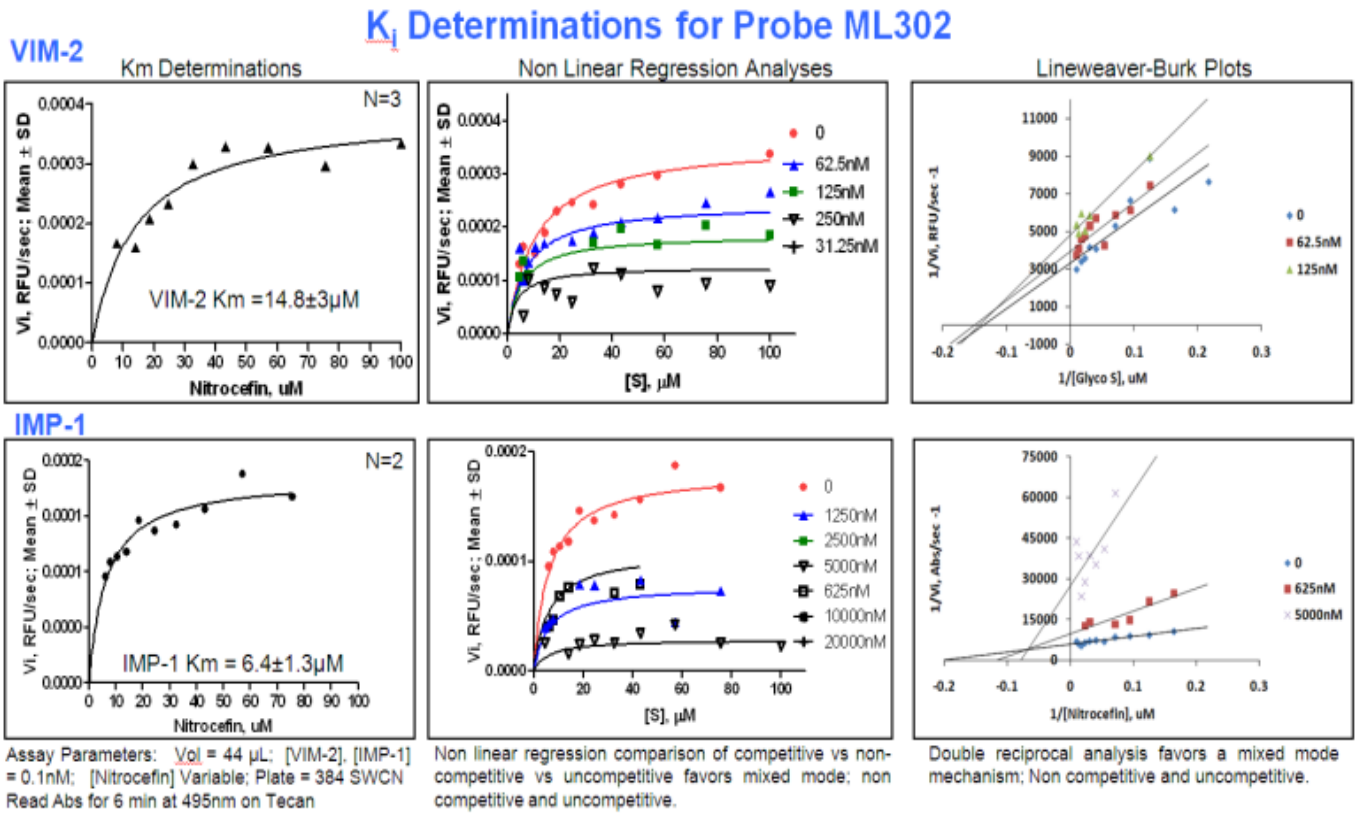
The assay provider has performed assays to elucidate the mode of inhibition and the probe's inhibitory constant (Ki) against VIM-2 and IMP-1. The results of these probe development efforts determined that probe ML302 is a non-selective, mixed mode inhibitor probe of the metallo-beta-lactamases VIM-2 and IMP-1 (**Figure 6**).

VIM-2 and IMP-1 Ki Assays (AID 624083 and AID 624084). The purpose of these assays is to determine the inhibition constant (Ki) and modality of probe candidate molecules. Kinetic assays were conducted by incubating a range of nitrocefin substrate concentrations (100nM - 5 µM) with varying inhibitor concentrations and 0.1 nM enzyme at room temperature in buffer containing 50mM HEPES, 50µM ZnSO4, 0.05% Brij 35, pH 7.1. Absorbance was measured on a Tecan Safire^2 monochromatic microplate reader at 495 nm. Initial velocities were obtained from plots of absorbance at 495 nm versus time, using data points from only the linear portion of the hydrolysis curve. Substrate hydrolysis was continuously monitored. Initial velocities were plotted vs. substrate concentration and kinetic parameters were calculated using Graphpad Prism version 5.01 suite of programs. Mode of inhibition was determined using fit comparison capability of Graphpad Prism version 5.01 and additionally evaluated by Lineweaver-Burke plot. K_i values were determined by non-linear regression analysis. The results of these studies demonstrated that probe ML302 (CID 53362017) is a mixed mode uncompetitive and non-competitive inhibitor, with a submicromolar VIM-2 (183 ± 24 nM) and IMP-1 (930 ± 97 nM) K_i values (**Figure 6**).

Table 5. *K_i values demonstrating ML302 is a mixed mode uncompetitive and non-competitive inhibitor*

Probe	Corp ID	SID	CID	Modality	Vim-2 Ki, nM	IMP-1 Ki, nM
New Probe ML302	SR-03000002555-2	134220672	53362017	Mixed mode Inhibitor	183	930
Prior Probe ML121	SR-01000775688-3	85856282	4870494	Non-competitive inhibition	148	ND

Figure 6. *Mixed mode of inhibition for VIM-2 and IMP-1*



4.3 Planned Future Studies. The assay provider plans to determine the efficacy of probe ML302 as well as prior probe ML121 in eukaryotic cells and in animal models of bacterial infection. Additional studies will explore the SAR around the rhodanine scaffold.

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