

Probe Report: Small Molecule That Targets AIP Binding Interactions in AIP-Dependent Bacterial Quorum Sensing.

Project Title: Small Molecule Inhibition of Staphylococcus Aureus Virulence
Grant Number: NIH 1 X01 MH078952-01

Screening Center Name: New Mexico Molecular Libraries Screening Center
Principal Investigator of Screening Center: Larry A. Sklar, Ph.D., University of New Mexico

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PubChem Assay Identifiers (AIDs): 527, 700, 1014, 1015, 1206 (Summary)

Assay or Pathway Target: AIP-Dependent Bacterial Quorum Sensing

Probe PubChem Compound Identifier (CID): 2333 (Benzbromarone)

Mode of Action: Inhibits binding of autoinducing pheromone (AIP) to cellular receptors.

Overview: Quorum sensing is a cell-to-cell communication system that permits members of a bacterial population to coordinate their behavior dependent on cell density. The mediators of this communication system are small, diffusible pheromones or autoinducers that are secreted by the bacteria and that accumulate extracellularly. At the appropriate concentration threshold that reflects a sufficient number or quorum of bacteria, the autoinducers signal gene expression programs that direct the coordinated switch of the population to a virulence-associated phenotype. Benzbromarone is a novel inhibitor of the RNAlII promoter activation response induced in *S. aureus* by autoinducing protein (AIP). Its inhibitory effects are elicited in the *S. aureus* RN6390 strain as well as in NM300, a local isolate that belongs to the community acquired methicillin resistant *S. aureus* clone USA300. It inhibits AIP-induced production of RNAlII transcripts and of virulence factors α -hemolysin and lipase. Receptor binding studies with FITC-AIP indicate that the primary mechanism of benzbromarone is to inhibit the binding of AIP to the AgrC cellular receptor, the initial step in the bacterial quorum sensing pathway. The IC_{50} for inhibition is in the range of 100 – 200 nM. Maximal effects can be achieved at 6 μ M concentrations in *in vitro* experimental systems. Cell viability is unaffected under these conditions for up to 20 hours at 37°C.

Assay Provider Information

Specific Aims:

Quorum sensing is a cell-to-cell communication system that permits members of a bacterial population to coordinate their behavior dependent on cell density (for review see ref. #1). The mediators of this communication system are small, diffusible pheromones or autoinducers that are secreted by the bacteria and that accumulate extracellularly. At the appropriate concentration threshold that reflects a sufficient number or quorum of bacteria, the autoinducers signal gene expression programs that direct the coordinated action of the population. The list of bacterial pathogens that use this method of communication to regulate virulence is expanding and now includes some of the most common bacterial pathogens of

humans including the medically important pathogen *Staphylococcus aureus*. (1). Because antibiotic resistance is an emerging problem in this pathogen (2) and vaccines are of limited efficacy (3), quorum sensing is becoming a therapeutic target for treatment of this infection (4-7). Attacking virulence by these strategies, termed "quorum quenching," has proven successful in two animal models of *S. aureus* infection (4, 7, and 8). We recently published data demonstrating that phagocyte-derived reactive oxidants inactivate the peptide thiolactone autoinducer of *S. aureus* and that this is important for host defense against this infection (8). Our data demonstrate that targeting virulence by chemical inactivation of the quorum sensing pheromone represents a viable treatment option. **We hypothesize that small molecule inhibitors of the peptide autoinducing pheromone (AIP) can abrogate virulence dependent gene expression.** To test this hypothesis, we are applying to the Molecular Libraries Screening Centers Network (MLSCN) to pursue the following specific aims:

Specific Aim #1: To screen libraries of small molecules in a high throughput fluorescence-based screening assay to identify compounds capable of suppressing pheromone-dependent activation of the promoter for a global regulator of *Staphylococcus aureus* virulence, RNAIII.

Specific Aim #2: To confirm that the compounds that inhibit RNAIII promoter activation also inhibit expression of the virulence genes that are regulated by RNAIII.

Significance:

Staphylococcus aureus permanently colonizes the moist squamous epithelium of the anterior nares of 20% of the population and is transiently associated with another 60%. Colonization is a known risk factor for invasive disease both in the hospital and the community and the resulting infections can range from superficial skin infections such as abscesses and impetigo to serious invasive infections such as septic arthritis, osteomyelitis, endocarditis, and toxic shock. Hospitalized patients who have been catheterized or who have undergone surgery are at increased risk of infection (9). Treatment of infections with antibiotics has become increasingly difficult owing to the widespread occurrence of strains that are antibiotic resistant including methicillin (formerly methicillin)-resistant *Staphylococcus aureus* (MRSA)(2, 10). Furthermore, the isolation of MRSA strains that are also resistant to vancomycin, the last drug for which the organism had been uniformly sensitive, raises the spectre of a return to the pre-antibiotic era. Moreover, treatment and care for patients with these infections results in significant costs to the health care system (11).

Of current concern is the emergence of community-acquired MRSA strains that are hypervirulent due in part to the expression of the Panton-Valentine Leukocidin (PVL) toxin that is associated with spontaneous skin and soft-tissue infections and necrotizing pneumonia (10, 12). Fatal community-acquired MRSA pneumonia and bacteremia was first reported in the United States among children in 1999 and in older adults with underlying diseases in 2005. However, it has now been described in immunocompetent young adults (10). Because vaccine trials have demonstrated limited efficacy for prevention of this infection and because this pathogen expresses virulence factors that subvert both innate and adaptive immunity (3, 9), there is considerable interest in developing new therapeutic agents for treatment of these more severe antibiotic-resistant infections. However, pharmaceutical companies have expressed minimal interest in attacking this problem (13) demonstrating the need for alternative mechanisms to screen compounds with antistaphylococcal activity. We believe that screening small molecule inhibitors of the genetic mechanism for upregulation of virulence factor production represents such a mechanism.

Rationale:

Targeting virulence by antagonism and/or inactivation of AIP. Structure-function studies of the *agr* peptide pheromones produced by *S. aureus* and the variations in the *agrC* receptor that binds these pheromones have determined the nature of the interactions and the sequences that are essential for activation and for inhibition of activation (6, 7, and 17-19). These studies have permitted synthesis of a universal peptide inhibitor (6) that inhibits RNAIII promoter activation both in vitro and in vivo (6, 7). In addition, our own work has demonstrated that oxidant modification of specific amino acids can inhibit binding and signaling through *agrC* (8). Together, these observations indicate that the receptor-ligand interaction that regulates quorum sensing dependent virulence is hydrophobic and that increases in polarity of the peptide can block binding. These data indicate the feasibility of inhibiting quorum sensing either by hydrophobic compound antagonism of peptide binding or by chemical modification of the peptide to prevent its binding.

Screening small molecule inhibitors of virulence for treatment of infectious disease.

The feasibility of treating infectious diseases by inhibition of virulence (13) has been experimentally verified (20-23). Compounds were screened from various libraries to identify small molecule inhibitors that either altered virulence gene expression (20, 23) or inhibited toxin activity (21, 22). Moreover, these small molecule inhibitors enhanced host defense during in vivo infection (21-23). These publications provide “proof of principle” for this approach and suggest that therapeutic drugs for the treatment of infection can be identified by this mechanism.

Background:

Substantial progress has been made in understanding the molecular mechanisms for virulence regulation, including quorum sensing, in this pathogen. Quorum sensing in *S. aureus* is regulated in part by the accessory gene regulator (*agr*) operon (14-16). The *agr* locus combines secretion of an autoinducing peptide thiolactone (AIP) ligand and a two component regulatory pathway to generate a regulatory RNA transcript RNAIII that is the effector of the operon. All *S. aureus* strains can be categorized into four groups based on the amino acid sequence of the AIP produced (1, 17). Whereas clinical isolates are enriched for type I AIP, all four groups are represented in human disease (14-16). Under conditions of high autoinducer concentration, i.e. high bacterial density, RNAIII downregulates gene expression encoding for surface adhesions while upregulating those encoding for capsule production, secreted toxins like PVL, proteases, and metabolic pathways (14, 15). The only protein product generated directly from RNAIII is delta hemolysin from the *hld* gene. This conversion from a tissue-adhering to a tissue-damaging and phagocyte-evading phenotype is thought to be important for in vivo pathogenesis and the development of invasive infection (4, 7, 8, and 14). *Agr* deletion mutants are less virulent in several animal models of infection including endocarditis, arthritis, pneumonia, and skin infection (14, 15). Direct demonstration of RNAIII production by bacteria in a lavage of a skin abscess (4, 8) indicates that quorum sensing is essential for acute infection at entry sites. Our recently published data suggest that phagocyte-derived reactive oxidants contribute to the control of *in vivo* quorum sensing in a skin infection model (8). Thus, the interaction of the pathogen with innate host defenses at the site of infection may dictate the type and course of the infection, i.e. acute and rapidly toxic vs. chronic and indolent. This raises the issue of how human infection occurs if the infecting strains secrete virulence pheromones that are inactivated by innate effectors. At

least two contributing factors that are not mutually exclusive could be involved: 1) these infections are opportunistic and occur primarily in hosts with defects in these innate effectors, and 2) the pathogen has mechanisms for producing virulence pheromones that can escape destruction by these effectors. Understanding all these aspects of the pathogenesis of staphylococcal infection, from the innate effectors that inhibit quorum sensing to the mechanisms that the pathogen uses to escape this control, are necessary to design drugs that target quorum sensing-dependent virulence.

Screening Center Information

Assay Implementation and Screening

PubChem Bioassay Name: Inhibitors of Bacterial Quorum Sensing

List of PubChem Bioassay Identifiers (AIDs): 527, 700, 1014, 1015

PubChem Primary Assay Description (AID 527):

A fluorescence based RNAIII promoter activation assay was developed using wild-type *agr* type 1 *S. aureus* that had been engineered with a plasmid encoding the promoter for RNAIII driving expression of GFP. When these reporter bacteria are in the early exponential phase of growth before quorum sensing has occurred, they are non-fluorescent. However, incubation with synthetic type 1 AIP induces GFP production and therefore fluorescence over the next 3 hours. Incubation of the reporter bacteria alone during this time frame is not sufficient to induce fluorescence indicating that minimal endogenous synthesis of AIP occurs under these conditions. The AIP activity is indicated by the intensity of GFP fluorescence.

Protocol. *Staphylococcus aureus* bacterial stocks SA RN6390: RNAIII-GFP (RNAIII), stored at -80 degrees C) are thawed and diluted with Columbia Broth (CB, Difco) to a final concentration of $\sim 2.5 \times 10^7$ cfu/ml. AIP is added to a final concentration of 0.1 microM and the bacteria/AIP cultures are added to wells of a 384-well plate at 18 microL/well. To each well is then added 2 microL of test compound (1 milliM stock in DMSO pre-diluted 1:50 in phosphate-buffered saline (PBS)) to result in final concentrations of 2 microM test compound and 0.2% (V/V) DMSO. Control wells are added with bacterial cultures containing AIP alone (AIP activation control or AAC), AIP plus the AIP inhibitor Erythrosin B (AIP inactivation control or AIC) and no additive (no activation control or NAC). Assay plates are then incubated 3 hours at 37 degrees C on a rocker. At the end of incubation, 40 microL of PBS containing 0.1% (V/V) Triton X-100 is added to each well. Well contents are mixed and plates subsequently stored on ice in the dark until analyzed by flow cytometry.

The HyperCyt high throughput flow cytometry platform (24,25) was used to sequentially sample cells from wells of 384-well microplates (2 microL/sample) for flow cytometer presentation at a rate of 40 samples/min. The resulting time-resolved data files were analyzed with IDLeQuery software (developed to support specialized analysis of time-resolved flow cytometry data files generated with the HyperCyt platform) to determine compound activity in each well.

The following data were obtained for each well: 1) the number of events analyzed, 2) the median fluorescence intensity of the bacteria, 3) the mean fluorescence intensity of the bacteria, 4) the % GFP negative bacteria, and 5) the % GFP positive bacteria. The number of events was used to assess the possibility of cytotoxicity mediated by the test compound, indicated by detection of less than 25% of the average number of events observed for all wells of a plate. The fluorescence intensity threshold for distinguishing %GFP positive bacteria was defined as the intensity level above which 90% of AAC control bacteria were observed.

Test compound inhibition of AIP activity was calculated by two methods. The first inhibition determination was on the basis of %GFP-positive cells (%I_GP) as follows:

$$\%I_GP = 100 \times [1 - (\%Pos_test - \%Pos_NAC) / (\%Pos_AAC - \%Pos_NAC)]$$

in which %Pos_test, %Pos_NAC, and %Pos_AAC represented the %GFP positive cells in wells containing test compound, the average %GFP-positive cells in AIP no activation control (NAC) wells and the average %GFP-positive cells in AIP activation (AAC) control wells, respectively. NAC and AIP inactivation control (AIC) results were routinely comparable in all assays.

The second inhibition determination was on the basis of mean GFP fluorescence intensity (%I_MFI) as follows:

$$\%I_MFI = 100 \times [1 - (MFI_test - MFI_NAC) / (MFI_AAC - MFI_NAC)]$$

in which MFI_test, MFI_NAC and MFI_AAC represented the mean GFP fluorescence intensity of cells in wells containing test compound, the average MFI of cells in NAC wells and the average MFI of cells in AAC wells, respectively.

Compounds were considered active in the primary screening assay if they resulted in 50% or greater inhibition for both fluorescence parameters.

Activity Score Calculation. The activity scores of the active compounds were calculated as:

$$\text{Activity Score} = 100 \times (\% \text{ Inhibition} - \%I_GP_Min) / (\%I_GP_Max - \%I_GP_Min)$$

in which %I_GP_Min and %I_GP_Max were 50 and 100, respectively. The calculated number was rounded up to the next higher integer value to obtain the final reported score. An activity score of 0 was assigned to all other tested compounds.

Comments. There were a number of wells in which the number of bacteria detected during analysis was less than 25% of the average number detected over all wells of the plate. This suggested that test compounds in these wells may have been cytotoxic. The word "Cytotoxic" is entered in the column labeled PUBCHEM_ASSAYDATA_COMMENT to flag these compounds.

Center Summary of the Primary Screen

A flow cytometry based high throughput screening (HTS) campaign was undertaken to identify novel small molecule inhibitors of the quorum sensing pathway of *Staphylococcus aureus*. A set of 9,993 compounds, designated the 10K Set Type 1 (10KST1), and a separate set of 16,322 compounds, designated the 17K Set Type 1 (17KST1), was obtained from the Molecular Libraries Small Molecule Repository (MLSMR) maintained by Discovery Partners International in conjunction with the NIH Molecular Libraries Screening Center Network. There was an overlap of 2,595 compounds common to the two sets. An additional 367 compounds were cherry-picked from the remainder of the MLSMR compound collection on the basis of structural similarities with active compounds detected in the first two screening sets. The total number of unique compounds evaluated was 24,087. Hit selection criteria were satisfied by 64 compounds (0.3%).

Probe Optimization

Description of secondary screens used to optimize probe structure.

1. Dose Response Hit Confirmation (AID 700).

The RNAlII promoter activation assay was used in a dose response format to further characterize the 64 hit compounds. For dose response curve application, test compound inhibition of AIP activity was calculated on the basis of mean GFP fluorescence intensity (%INHIB_MFI) as follows:

$$\%INHIB_MFI = 100 \times [1 - (MFI_test - MFI_NAC)/(MFI_AAC - MFI_NAC)]$$

in which MFI_test, MFI_NAC and MFI_AAC represented the mean GFP fluorescence intensity of cells in wells containing test compound, the average MFI of cells in NAC wells and the average MFI of cells in AAC wells, respectively.

Each compound was tested at 9 concentrations ranging from 3 nM to 20 μ M. The concentration that inhibited activity by 50% (IC₅₀) was used as the basis of distinguishing active and inactive test compounds in the dose response assay. Test compounds with IC₅₀ less than 10 μ M were scored as active and all others as inactive. 19 compounds were confirmed as active by these criteria.

2. Primary screening of chemotype families in a structure activity relationship (SAR) series of compounds (AID 1014)

Nine major chemotype families representing the most potent inhibitors were selected for follow up structure activity relationship (SAR) evaluation. A set of 240 compounds was selected by application of computational similarity search techniques to a subset of the ChemDiv collection of ~ 700,000 compounds. The compounds were purchased and screened in the RNAlII promoter activation assay in HTS format as described above. There were 32 compounds that satisfied hit selection criteria (13.3%).

3. Dose Response Confirmation of SAR Series Hits (AID 1015)

The RNAIII promoter activation assay was used in a dose response format as described above to further characterize the 32 hit compounds. The 32 compounds represented 7 of the 9 tested chemotype families and all were confirmed as active. Four of the active compounds were in Family 2, the chemotype family of the probe described in this report. The dose response profile of the probe, benzbromarone, is illustrated in Fig. 1.

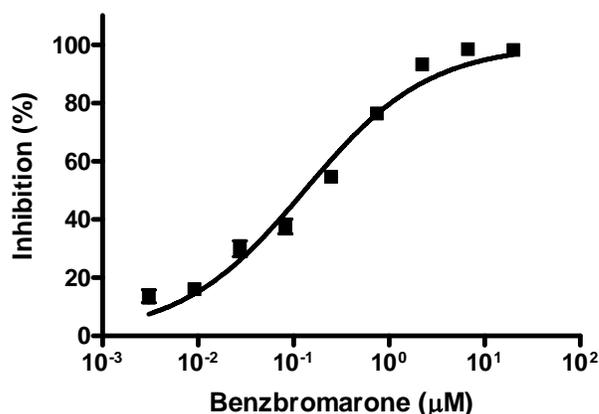


Fig. 1. Dose response profile of benzbromarone in the RNAIII promoter activation assay. $IC_{50} = 0.13 \mu M$ in this experiment.

4. Viability Assay. An important characteristic of a quorum sensing pathway probe is that it not affect bacterial viability. *S. aureus* RN6390 was therefore cultured for 3 or 20 hours in the presence and absence of benzbromarone. There were no detectable effects on the resulting viable colony counts in either the short or long term cultures (Fig. 2). This was consistent with the lack of benzbromarone effects on the numbers of bacteria detected in the flow cytometry based RNAIII promoter activation assay.

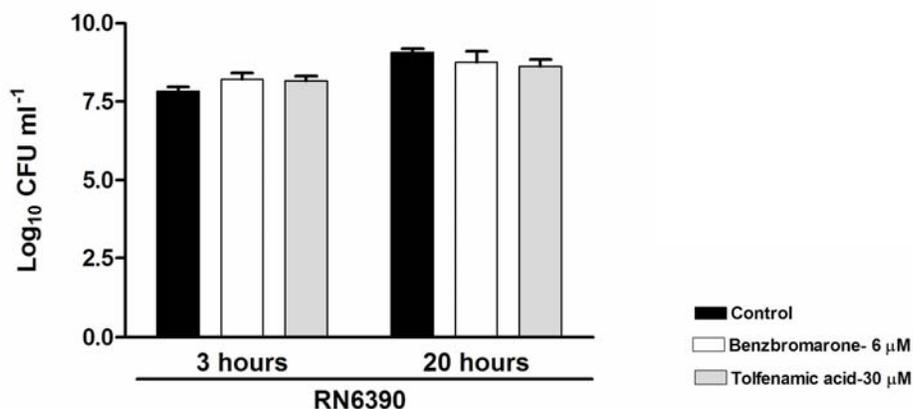


Fig. 2. Benzbromarone does not affect cell viability.

5. RNAIII RT-qPCR Assay. As an alternative approach to confirm benzbromarone effects on RNAIII promoter activation, RNAIII RT-qPCR assays were performed on total RNA isolated from *S. aureus* cultures 3 hours after addition of exogenous AIP, as previously described (26). In the presence of 6 μM benzbromarone RNAIII levels were reduced more than 90% in comparison to control cells cultured in its absence (Fig. 3). This was observed in *S. aureus*

RN6390 and was even more pronounced in *S. aureus* NM300, a local isolate that belongs to the community acquired methicillin resistant *S. aureus* (CA-MRSA) clone USA300 (provided by Dr. Steve Young, Department of Pathology, University of New Mexico), associated with spontaneous skin and soft tissue infections and necrotizing pneumonia (10).

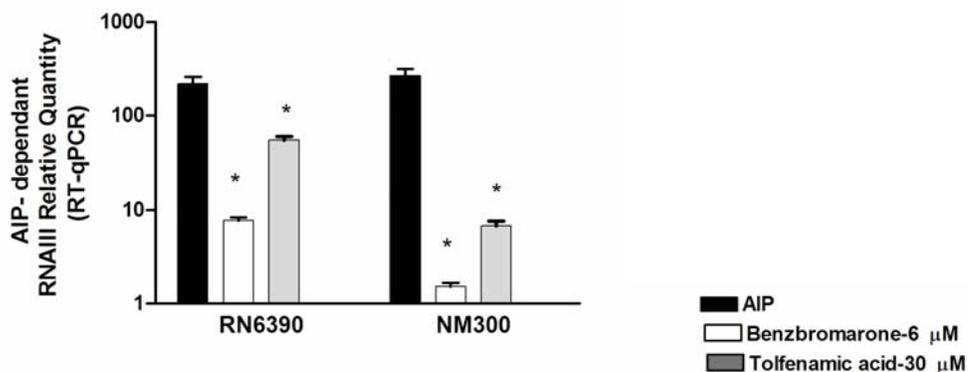


Fig. 3. Benzbromarone reduces AIP-induced RNAIII transcript production in two *S. aureus* strains.

6. Virulence Factors Induced by AIP. Upregulation of RNAIII production leads *S. aureus* to switch to an invasive phenotype that secretes a variety of virulence factors such as α -hemolysin and lipase. α -hemolysin activity was measured in the supernatants of *S. aureus* RN6390 and NM300 strains 6.5 hours after AIP treatment on the basis of rabbit erythrocyte lysis, as previously described (27,28). A hemolytic unit (HA50) was defined as the amount of test solution able to liberate half of the total hemoglobin from the erythrocyte (28). α -hemolysin activity was abrogated in both strains in the presence of 6 μM benzbromarone (Fig. 4).

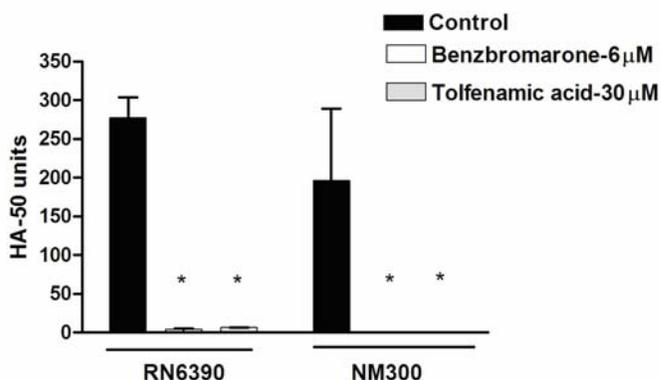


Fig. 4. Inhibition of AIP-induced α -hemolysin production by benzbromarone.

Lipase (glycerol ester hydrolase) production was evaluated by monitoring the lipolytic kinetics of an overnight culture supernatant on tributyrin emulsion at wavelength 450nm as previously described (29). AIP-induced lipase activity was eliminated in both strains by benzbromarone (Fig. 5).

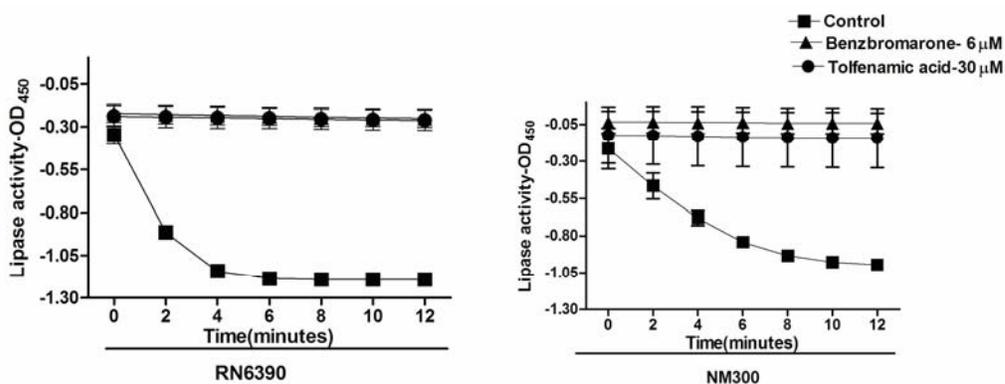


Fig. 5. Inhibition of AIP-induced lipase production by benzbromarone.

6. FITC_AIP Binding Assay

To determine benzbromarone effects on AIP binding to its *AgrC* surface receptor, a fluorescein-conjugate of AIP (FITC-AIP) was prepared. *S. aureus* cultures were incubated with 1 μM FITC-AIP for 1 h at 37°C in the presence and absence of 6 μM benzbromarone. AIP-FITC binding was reduced more than 60% by benzbromarone in both *S. aureus* strains (Fig. 6).

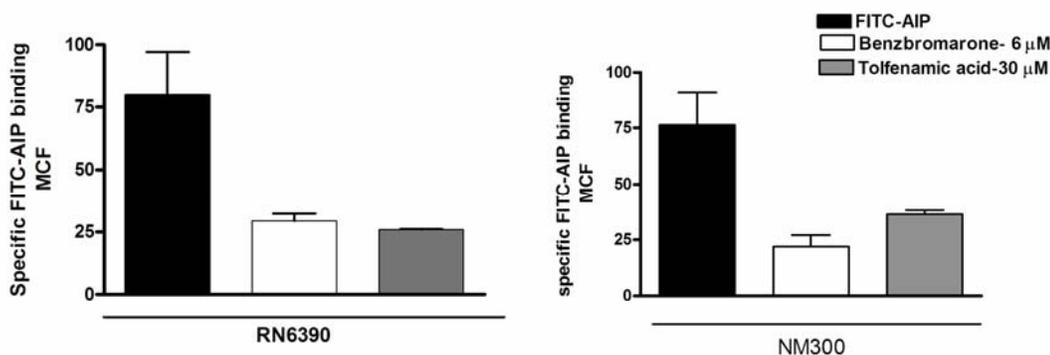
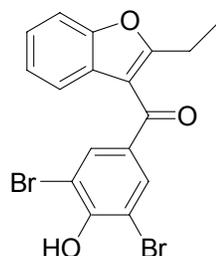


Fig. 6. Benzbromarone inhibits binding of FITC-AIP to *S. aureus*.

Center comments on chemistry strategy leading to probe identification:

Characterization data for Benzbromarone



(PubChem CID: 2333)

White solid, mp 129-130°C; ^1H NMR (300 MHz, CDCl_3) δ 7.98(s, 2H), 7.51-7.47(m, 1H), 7.43-7.39(m, 1H), 7.33-7.20(m, 2H), 6.43(s, 1H), 2.90(q, $J = 7.63, 7.49\text{Hz}$, 2H), 1.36(t, $J = 7.63, 7.48\text{Hz}$, 3H). ^{13}C NMR (75 MHz, CDCl_3) δ 187.78, 166.40, 153.65, 153.11, 133.65, 133.42, 126.50, 124.61, 123.76, 120.93, 115.32, 111.11, 109.98, 21.92, 12.22. FTIR (KBr, cm^{-1}) 2967(w), 1616(s), 1571(m), 1453(m), 755(s).

HPLC-MS

Benzbromarone was eluted from a Waters Symmetry® C_{18} 5 μm 3.0 X 150mm column with 60-90 % $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ containing 0.01% formic acid, $R_t = 13.45$ min. (Fig 10). UV-Vis at $R_t = 13.45$ min. λ_{max} 276 nm (Fig. 11). ESI-MS m/z (ES+) calcd for $\text{C}_{17}\text{H}_{12}\text{O}_2\text{Br}_2$ (M+H) $^+$ 422.92, found 422.83 (Fig. 11).

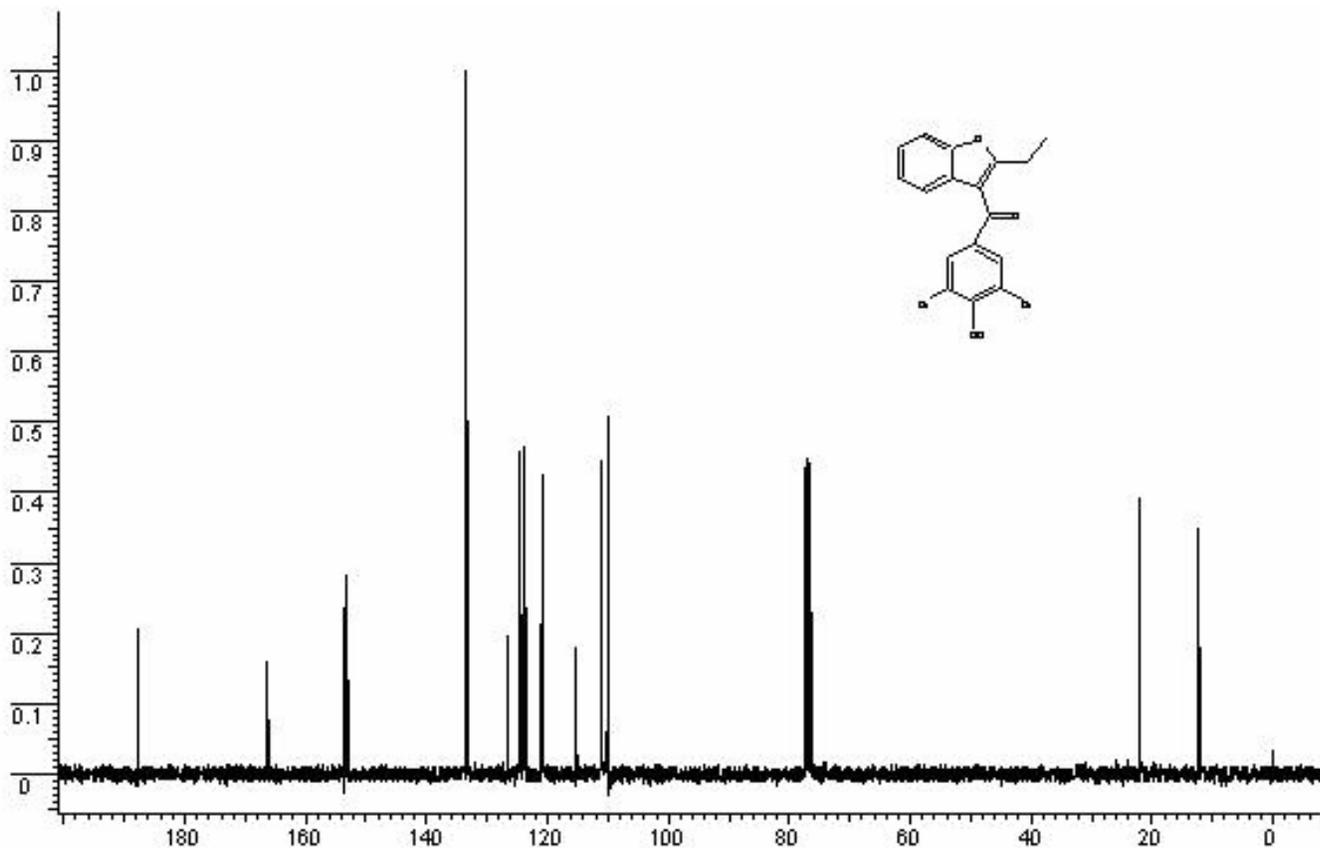


Fig.7. ^{13}C NMR of Benzbromarone.

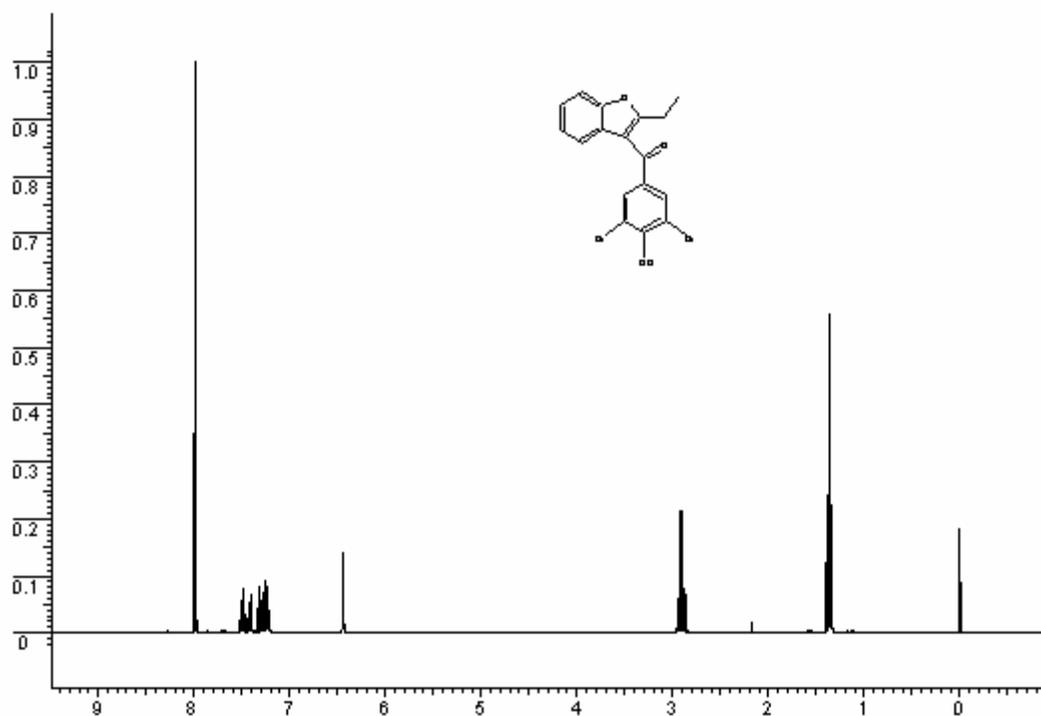


Fig.8. ^1H NMR of Benzbromarone.

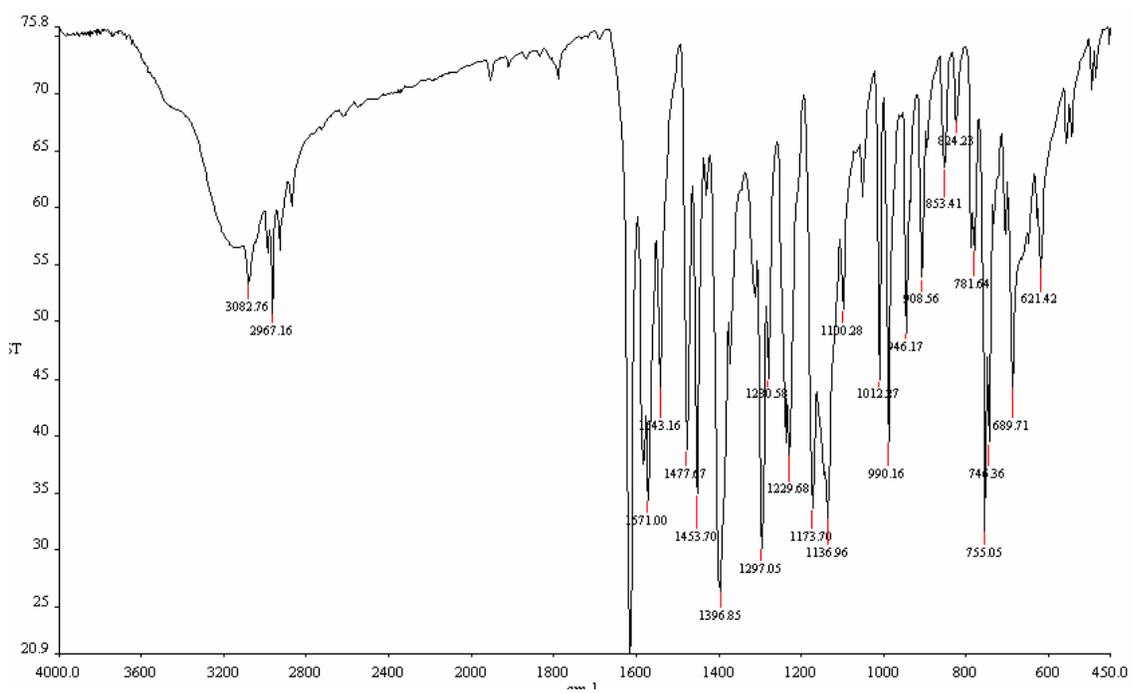


Fig. 9. FTIR Spectrum of Benzbromarone

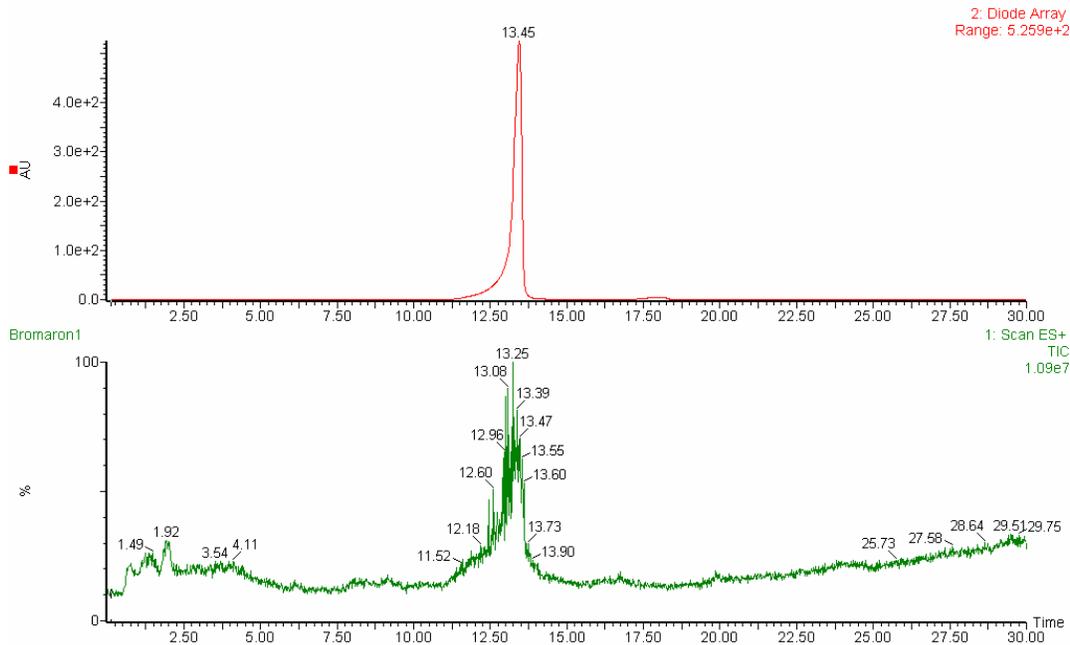


Fig. 10. HPLC PDA (top) and ESI-MS (ES+) TIC (bottom) of Benzbromarone.

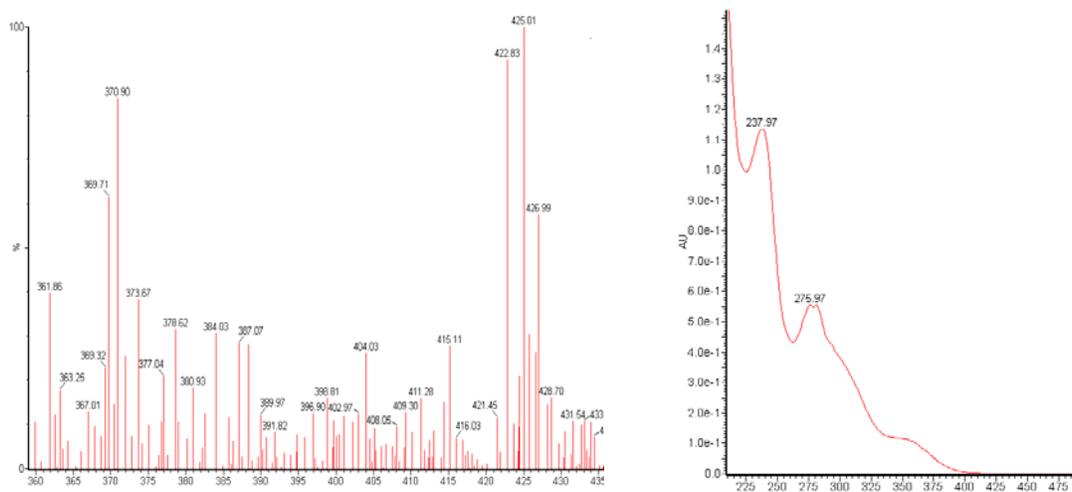


Fig. 11. ESI-MS (left) and UV-Vis (right) of Benzbromarone.

Center summary of probe properties and recommendations for the scientific use of probe as research tool.

Benzbromarone is a novel inhibitor of the RNAIII promoter activation response induced in *S. aureus* by AIP. Its inhibitory effects are elicited in the RN6390 strain as well as on NM300, a local isolate that belongs to the community acquired methicillin resistant *S. aureus* clone USA300. It inhibits AIP-induced production of RNAIII transcripts and of virulence factors α -hemolysin and lipase. Receptor binding studies with FITC-AIP indicate that the primary mechanism of benzbromarone is to inhibit the binding of AIP to the AgrC cellular receptor,

the initial step in the bacterial quorum sensing pathway. The IC_{50} for inhibition is in the range of 100 – 200 nM. Maximal effects can be achieved at 6 μ M concentrations in *in vitro* experimental systems. Cell viability is unaffected under these conditions for up to 20 hours at 37°C.

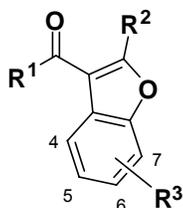
Appendices

Comparative data on probe, similar compound structures and prior probes.

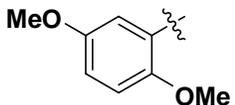
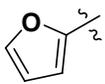
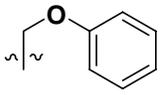
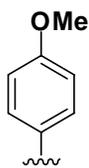
Structure Activity Relationship (SAR) Evaluation

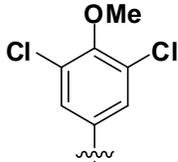
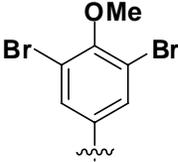
The relatively high activity of benzbromarone (Entry 1), lead to scaffold exploration of the benzofuranyl ketone scaffold. SAR by commerce around family 2 was based around changes at regions 1, 2, and 3. Table 1 depicts the structural changes represented for this family. Region 1 was explored with other aryl substituents as well as alkyl and ester moieties. Activity was predominantly observed with aryl substituents (Entries 1-3). However, it should be noted that not all permutations of the other two regions were represented while holding region 1 constant as an aryl group. Entry 8 is lacking an aryl group altogether and still shows activity. A *para*-hydroxy seems to be desirable for activity in this region with the di-bromo substituted benzbromarone maintaining its role as the best inhibitor. No analogs with a methyl at Region 1 showed activity while Entry 8 was active as the ethyl ester. No other esters were active despite a good range of variation in regions 2 and 3, nor was the one example of a free carboxylic acid (Entry 18). Region 2 was varied with alkyl and aryl substituents, with only the alkyl analogs (Entries 1-3 and 8) demonstrating activity. No unsubstituted Region 2 compounds showed any activity. Region 3 benzofuran, aryl substitutions yielded only one active compound, again, Entry 8. Similar to the substitution in Entry 1 (Region1), Entry 8 seemed to gain some activity from the sterically bulky dibromo-methoxy substitution.

Table 1.



Entry	ID (SID)	R ¹	R ²	R ³	Activity (μM)
1	Benzbromarone (24424558)		Et	H	0.13
2	3039-0682 (26746701)		ⁿ Bu	H	0.54
3	Benzarone (24424590)		Et	H	1.29
4	6526-0210 (26746736)		H	5-hydroxy	-

5	8005-2108 (26746682)		H	5-hydroxy	-
6	8003-0139 (26746749)		H	5-hydroxy	-
7	8009-4552 (26746758)	Me	Me	5-hydroxy	-
8	8015-2241 (26746675)	-OEt	Me	4,6-dibromo-5-methoxy	0.78
9	4356-0440 (26746684)	-OEt	Me	5-hydroxy	-
10	3341-4855 (26746705)	-OEt	Me	6-bromo-4-formyl-5-hydroxy	-
11	3570-0599 (26746708)	-OMe	Me	6-bromo-5-hydroxy	-
12	4356-0227 (26746717)	-O ⁱ Pr	Me	5-hydroxy	-
13	3473-2725 (26746686)	-OEt		5-hydroxy	-
14	1988-1470 (26746698)	-OEt	Ph	5-hydroxy	-
15	5084-0001 (26746722)	-OEt		5-hydroxy	-

16	4546-0015 (26746720)	-OEt		5-hydroxy	-
17	4546-0014 (26746719)	-OEt		5-hydroxy	-
18	3498-0008 (26746707)	-OH	Me	5-hydroxy	-

Several other closely related analogs were also screened (Figure 1), none of which showed any activity. Compound 19 (SID 26746731) is a tricyclic analog of the Region 2 methyl derivatives while compounds 20 (SID 26746673), 21 (SID 26746672), and 22 (SID 26746683) are regioisomers of the scaffold where the benzofuranyl ketone substitution is at ring position 2. Compounds 23 (SID 26746721) and 24 (SID 26746723) are similarity matches of the scaffold which also showed no apparent activity.

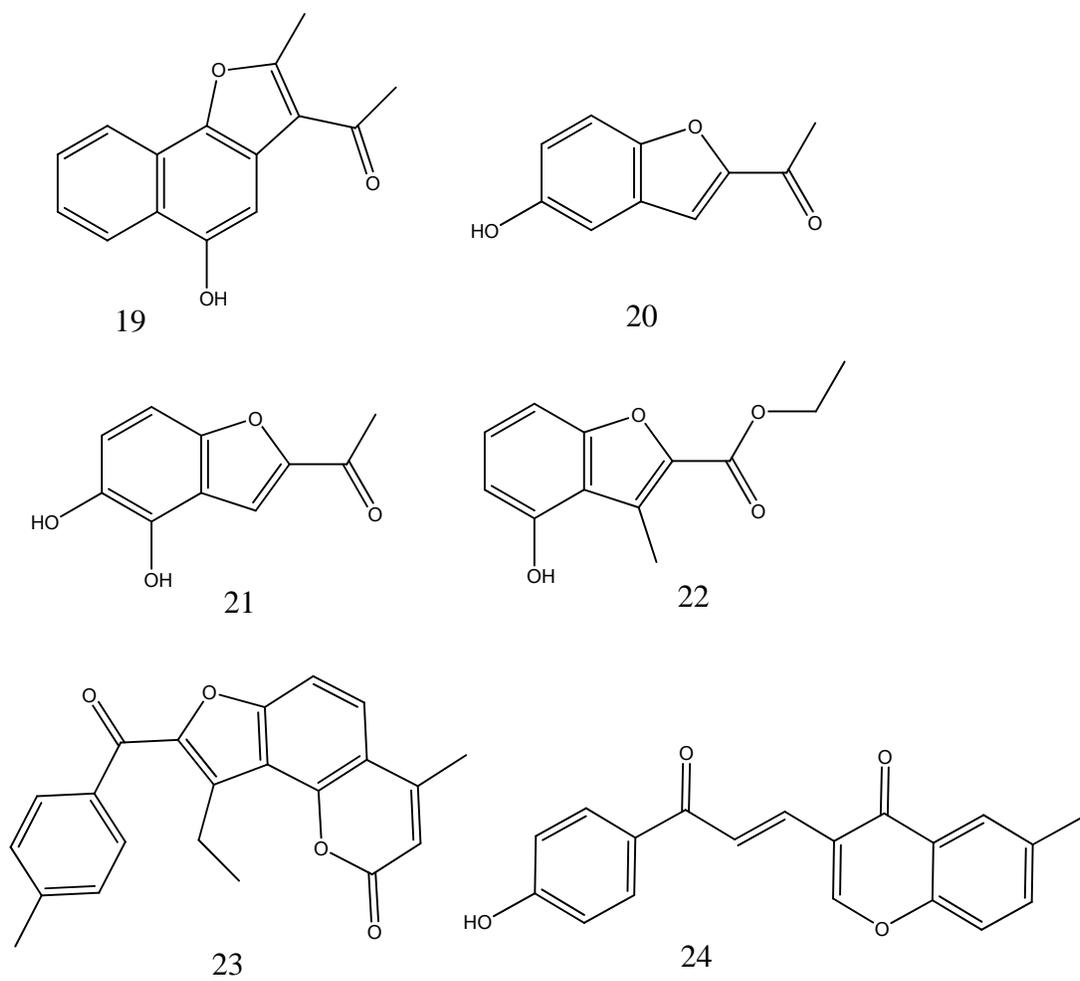


Figure 1. Inactive Family 2 analogs.

Comparative data showing probe specificity for target

Benzbromarone is a uricosuric that acts by increasing uric acid clearance. It is used in the treatment of gout. It has been analyzed in 286 assays published on the PubChem website and confirmed to be active in 10 (Table 2). Two were bacterial quorum sensing assays described in this report. Others include assays of chemicals that shorten yeast life span (2), S1P1 agonists and agonism potentiators (2), cytochrome P450 inhibitors (2), antagonists of a hypoxia response element signaling pathway (1), and inhibitors of HSD17B4, hydroxysteroid (17-beta) dehydrogenase 4 (1).

Table 2. Benzbromarone activity reported in confirmation assays

AID	Active	Inactive	Tested	Outcome	Method	Name
410	4177	618	4870	Confirmatory		p450-cyp1a2
466	266	133	399	Confirmatory		Dose Response Assays for S1P1 Agonists and Agonism Potentiators - Potentiator Assay 60K MLSMR
468	281	118	399	Confirmatory		Dose Response Assays for S1P1 Agonists and Agonism Potentiators - Agonist Assay 60K MLSMR
700	19	50	69	Confirmatory		Dose Response Assay for Inhibitors of Bacterial Quorum Sensing
849	480	235	715	Confirmatory		Screen for Chemicals that Shorten Yeast Lifespan, Dose response
850	468	247	715	Confirmatory		Screen for Chemicals that Shorten Yeast Lifespan, Dose Response Permissive Growth Control
883	1275	2940	4970	Confirmatory		qHTS Assay for Inhibitors and Substrates of Cytochrome P450 2C9
893	5650	5759	12411	Confirmatory		qHTS Assay for Inhibitors of HSD17B4, hydroxysteroid (17-beta) dehydrogenase 4
915	442	2767	4650	Confirmatory		qHTS Assay for Identification of Small Molecule Antagonists for Hypoxia Response Element Signaling Pathway
1015	32		32	Confirmatory		Inhibitors of Bacterial Quorum Sensing Structure Activity Relationship (SAR) Analysis: Dose Response Assay

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