High-Throughput Screening of the Natural Product Discovery Fraction Library from the National Cancer Institute Identified Fractions with Selective Antimicrobial Activity

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Introduction

- New antimicrobials are needed to combat bacterial and fungal infections caused by resistant pathogens
- Most clinically important antimicrobial classes were initially discovered from natural products
- A high-throughput, *in vitro*, cell-based screen (HTS) of the NCI Program for Natural Product Discovery (NPNPD) natural product fraction library was completed to help identify novel chemical matter with antimicrobial activity
  - The HTS was conducted by the National Institute of Allergy and Infectious Diseases (NIAID), JMI Laboratories, and the National Cancer Institute (NCI)

Materials and Methods

- **Figure 1 displays a high-level view of the HTS**
- **Chemical library**
  - NCI Program for Natural Product Discovery (NPNPD) prefractionated library
    - Described in Thornburg et al. (2018) [1]
  - Composed of 40,832 crude natural product extracts
    - Collected from a variety of global sites and organism sources (Figure 2A)
    - Each extract was partially purified by NCI into 7 fractions (Figure 2B)
    - 326,656 total samples in groups of 8 by extract were arrayed in 384-well plates
    - Each fraction was a mixture of components
  - Proof-of-concept subfractionation phase
    - 75 initial HTS hit fractions were selected by NCI (Figure 1)
    - These 75 hit fractions were each purified into 22 subfractions (Figure 2C)
    - Each subfraction was screened against the strain panel for growth inhibition
- **HTS strains**
  - *Staphylococcus aureus* ATCC 29213 (Gram-positive; MSSA; [2])
  - *Escherichia coli* BW25113 (Gram-negative; efflux competent; [3]; obtained from the Coli Genetic Stock Center [CGSC] New Haven, CT)
  - *Escherichia coli* JW5503-1 (Gram-negative; *tolC* efflux deficient; [3]; obtained from the CGSC)
  - *Candida albicans* ATCC 90028 (yeast; [4])
- **HTS process (Figure 1)**
  - *In vitro*, cell-based, growth inhibition of bacterial and fungal strains
  - Media: CAMHB (bacterial strains) and RPMI 1640 (fungal strain); 2% DMSO final
  - Format: 384-well plate; 50 µL final well volume
  - Readout: alamarBlue (Bio-Rad) fluorescent growth signal at 590 nm in a Tecan Spark reader
  - Control antimicrobials: ampicillin, levofloxacin, and amphotericin B
  - Z' was ~0.8 for all 4 strains [5]
  - Screening concentrations
    - Single point (10 mg/L total fraction) with ≥2 replicates vs. 4 strains
    - Follow-up dose response testing in duplicate for initial hits: 0.08–10 mg/L total fraction
- **Proof-of-concept subfractionation testing (Figure 1)**
  - Same methods as HTS assay, except
    - 96-well format; 100 µL final well volume
    - Readout: Minimal Inhibitory Concentration (MIC) testing conducted according to CLSI methods [2, 4, 6, 7]
      - OD_{600} (in place of alamarBlue fluorescent signal)
      - Dose-response range: 0.005–10 mg/L

Results

- **Initial single-concentration screening (Figure 1)**
  - Figure 3 displays the replicate % inhibition data (at 10 mg/L) for the 4 strains
  - Numerous actives were identified (Table 1)
    - 9,524 initial actives were selected for testing in a dose-response format
- **Dose-response screening of initial actives (Figure 1)**
  - Table 2 displays the confirmed hit fractions by species and extract source (some fractions were hits against >1 species)
    - *C. albicans*: 2,590 hit fractions (62% from plant sources; 0.8% overall hit rate)
    - *S. aureus*: 734 hit fractions (72% from marine sources; 0.2% overall hit rate)
    - *E. coli* (*tolC*): 682 hit fractions (65% from marine sources; 0.2% overall hit rate)
    - *E. coli* (wild-type): 140 hit fractions (95% from marine sources; 0.04% overall hit rate)
  - Table 3 presents data on several specific categories of hits (e.g., pan inhibitors or *S. aureus*-specific inhibitors)
    - In many cases, 1 extract was associated with ≥2 hit fractions
  - Figure 4 displays growth-inhibition curves for examples of 4 categories of hit fractions
- **Proof-of-concept subfractionation of hit fractions and retesting (Figure 1)**
  - Each initial library fraction was composed of ~20 molecular species [1]
  - To begin the pilot deconvolution process, 75 initial hit fractions were selected for further fractionation by NCI and each subfraction was tested for inhibition in single-concentration (10 mg/L) growth assays (Figure 2C)
  - ~80% of the expected antimicrobial activities for 75 hit fractions were further narrowed to ≥1 subfraction(s) (Figure 5)
  - 24 active subfractions were selected and 87.5% of these displayed their expected activity by MIC assay (Figure 6)

Table 1. Number of fractions categorized as hits at 10 mg/L using three criteria against each strain

	Hit criterion 1 [a]	Hit criterion 2 [b]	Hit criterion 3 [c]
C. albicans ATCC 90028	5,084	NA	4,293
E. coli BW25113 (wild-type)	1,447	20	157
E. coli JW5503-1 (*tolC*)	1,504	843	478
S. aureus ATCC 29213	1,646	305	1,040
Total	8,457 [d]	1,067 [d]	5,175 [d]

NA, not applicable
[a] Any well that led to a %I value ≥ (mean (µ) %I + 4 standard deviations (σ)) for ≥50% of the replicates (some fractions were screened >2 times).
[b] Any well not identified by hit criterion 1 that displayed %I values ≥ (µ + 3σ) for ≥2 data replicates (some fractions were screened >2 times).
[c] Any well that exhibited ≥70% inhibition for ≥2 data replicates (some fractions were screened >2 times).
[d] Some wells were categorized as hits against more than 1 strain but were only counted once in the total.

Table 2. Summary of the number of hits and extract sources identified against the four screening strains after HTS dose response testing

		Extract source (number and percent of fractions)			
Fractions in complete library		**Marine (C)**	**Plant (N)**	**Microbial (F)**	**Algal (J)**
326,656		73,920 (23%)	247,808 (76%)	3,520 (1%)	1,408 (0.4%)
Strain	**No. of hit fractions [a]**				
C. albicans	2,590	943 (36%)	1,612 (62%)	33 (1%)	2 (0.1%)
E. coli (wild-type)	140	133 (95%)	5 (4%)	2 (1%)	0
E. coli (*tolC*)	682	446 (65%)	220 (32%)	14 (2%)	2 (0.3%)
S. aureus	734	531 (72%)	167 (23%)	36 (5%)	0

[a] Fractions displaying dose-response data with ≥1 replicate with an IC_{50} value ≤7.5 mg/L and R^2 value ≥0.8 or with an IC_{50} value ≤0.1 mg/L without regard to the R^2 value. These totals include some fractions that were not categorized as hits against a specific strain by single-concentration screening but were categorized as hits against that same strain in dose responses.

Table 3. Summary of the number of hits identified for specific strain subsets after dose response testing

		No. of fractions categorized as hits by extract [a]							
Strain subset	**No. of hit fractions [b,c]**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Pan inhibition (all 4 strains)	118	37	12	10	3	3	0	0	0
C. albicans only	1,772	793	220	100	39	14	1	1	0
E. coli (wild-type) only	0	NA	NA	NA	NA	NA	NA	NA	NA
E. coli (wild-type) and *E. coli* (*tolC*) only	0	NA	NA	NA	NA	NA	NA	NA	NA
E. coli (*tolC*) only	85	75	5	0	0	0	0	0	0
S. aureus only	77	47	6	4	0	0	1	0	0
S. aureus and *E. coli* (*tolC*) only	39	33	3	0	0	0	0	0	0
E. coli (wild-type), *E. coli* (*tolC*), and *S. aureus* only	2	2	0	0	0	0	0	0	0
Any bacterial strain but not *C. albicans*	264	198	21	6	0	0	1	0	0

NA, not applicable
[a] The columns 1–8 display sums of hits by extract and do not represent the specific fraction numbers.
[b] Fractions displaying dose-response data with ≥1 replicate with an IC_{50} value ≤7.5 mg/L and R^2 value ≥0.8 or with an IC_{50} value ≤0.1 mg/L without regard to the R^2 value.
[c] These hit sets (except the pan-inhibitor set) were additionally filtered by removing any fraction that exhibited any on-scale IC_{50} value against any non-set strain.

Figure 1. Overview of the process and results from the NCI NP prefractionated library antimicrobial HTS. The main HTS consisted of screening each fraction at a single concentration followed by testing each active fraction in a dose-response format. As a proof-of-concept, 75 hit fractions were selected, each purified into 22 subfractions, and tested at 10 mg/L against the 4 strains (see Figure 5). A small set of subfractions with confirmed activity was also tested in the dose-response format (see Figure 6).

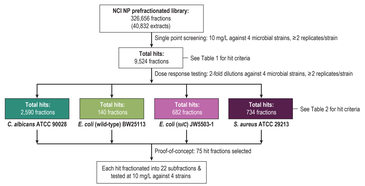


Figure 2. Structure of the NCI prefractionated natural product library. (A) The library was composed of 40,832 extract mixtures from various sources. (B) For each crude extract (0), the library also contained 7 semi-purified fractions (1–7). In this hypothetical example, the starting extract and fraction 2 displayed antimicrobial activity (orange). (C) Hit fraction 2 was further purified into 22 additional subfractions. In this example, the antimicrobial activity was further purified into subfraction 5. (D) Fraction nomenclature.

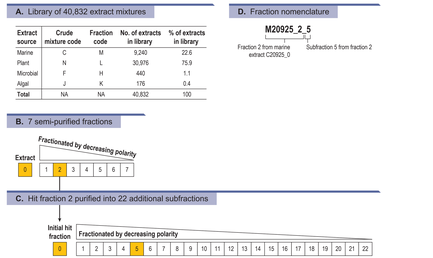


Figure 3. Hit landscapes for all 4 strains from the initial replicate, single-concentration screening. Each library fraction was screened in duplicate at 10 mg/L for growth inhibition against (A) *C. albicans* ATCC 90028; (B) *S. aureus* ATCC 29213; (C) *E. coli* (wild-type) BW25113; and (D) *E. coli* (*tolC*) JW5503-1. The replicate % inhibition (I) values are plotted in the XY plane with frequencies plotted along the Z axis. Fractions that exhibited strong growth inhibition in both replicates are clustered in the upper right-hand corner of each plot.

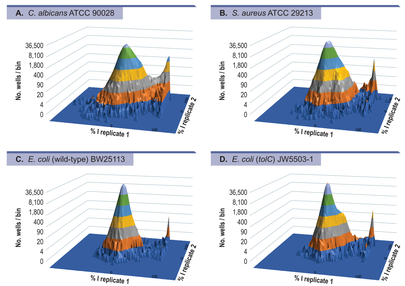


Figure 4. Dose-response curves for 4 hit fractions. The data display the % inhibition of growth at various concentrations of the library fraction against the 4 strains. Different inhibition phenotypes were observed in these examples. Each fraction was a semi-purified mixture of various molecular species.

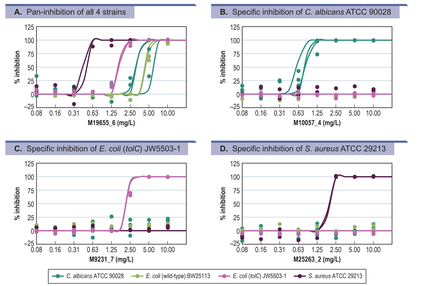


Figure 5. Heat maps demonstrating partitioning of antimicrobial activity after further purification of NCI NP library hit fractions [8]. The 75 active fractions that were selected after the primary HTS were further subdivided into 22 subfractions, which were then screened against the 4-strain panel at 10 mg/L. The growth-inhibition results for all 22 subfractions of 10 initial hits are displayed. The fraction names are shown at the top. Fraction 0 corresponded to the original hit fraction identified in the HTS. Orange shading in the second row indicates which strains were categorized as hits during the main HTS. CA = *C. albicans* ATCC 90028; EC = *E. coli* (wild-type) BW25113; SA = *S. aureus* ATCC 29213; TC = *E. coli* (*tolC*) JW5503-1.

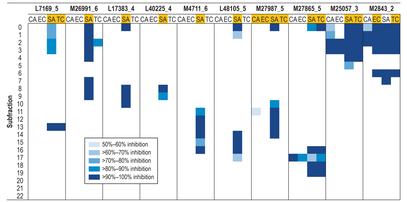
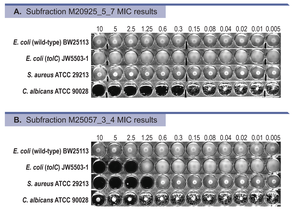


Figure 6. Examples of MIC activity for 2 purified active subfractions. These panels display the results of MIC experiments for subfraction M20925_5_7 (A) and M25057_3_4 [see Figure 5] (B) against all 4 test strains. The subfractions were derived by further purification of active fractions identified during the HTS. The concentration of the subfraction material in each well (mg/L) is indicated at the top of each panel. Further characterization of the hit subfractions is ongoing.



Conclusions

- Many hit fractions with antibacterial and/or antifungal activity were identified from the NCI natural product prefractionated library
- Various classes of hit fractions were identified, including *C. albicans*-specific inhibitors (1,772), pan-species inhibitors (118), *E. coli* (*tolC*)-specific inhibitors (85), and *S. aureus*-specific inhibitors (77)
  - No inhibitor fractions were found that specifically inhibited only the *E. coli* wild-type and *E. coli tolC* strains
- In a proof-of-concept deconvolution study, antimicrobial activity often confirmed and mapped to 1 or more subfractions; additional deconvolution experiments are underway
- The hits have not yet been purified to single molecular species and no mode-of-action studies have been conducted
- This set of hit fractions potentially represents promising novel antimicrobial chemical matter
- These data and materials are available to support further research
  - Please contact the NCI Natural Products Branch at ncinatprodrep@mail.nih.gov for additional information

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