SUPPLEMENT

Rapid, semi-quantitative assay to discriminate among compounds with activity against replicating or non-replicating *Mycobacterium tuberculosis*

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Running title: Charcoal agar resazurin assay (CARA) for Mycobacteria

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FIGURE LEGENDS, SUPPLEMENT:

Fig. S1. (**a**) The addition of 0.4% (wt/vol) activated charcoal to 7H9-glycerol liquid medium prior to autoclaving decolorizes the medium. (**b**) Evidence that activated charcoal binds Alamar blue in solution. Stationary-phase Msm in 7H9-glycerol liquid medium (200 μL) at an OD_{580} of 0.5 was mixed with 0 – 0.4% activated charcoal in a 96-well plate. Alamar blue:Tween 80 (in PBS) (1:1) (40 μL) was added, and fluorescence allowed to develop, during 1 hour at room temperature. Data are means +/- standard deviation of quadruplicate wells. In some cases, error bars are obscured by data points. This is one of 3 experiments with similar results. Colonies arising from 10-fold serial dilutions of wild-type Mtb were observed at similar times when grown on (**c**) 7H11-OADC agar or (**d**) 7H11-OADC agar supplemented with 0.4% activated charcoal. Colonies were often smaller on 7H11-OADC-charcoal plates.

Fig. S2. Correlation between the numbers of inoculated Msm on CARA plates and Alamar blue fluorescence. CARA plates were incubated at 37 °C for either 24 (**a**) or 48 (**b**) hours. Points represent the average of 8 replicates +/- standard deviation. Graphs are representative examples of 2 independent experiments.

Fig. S3. Examples of compounds whose CFU counts were not significantly impacted by addition of activated charcoal to agar plates used to enumerate surviving bacilli. Inclusion of 0.4% (wt/vol) activated charcoal in 7H11-OADC agar plates had little impact on CFU counts for replicating and non-replicating wild-type Mtb treated with kanamycin (**a** and **b**) or moxifloxacin (**c** and **d**). Data are means +/- standard deviation for 1 (kanamycin) and 3 (moxifloxacin) experiments. Numbers

above data points are p-values (unpaired Student's t-test) compared to the DMSO-treated control, and color-coded (blue = no charcoal; red = plus charcoal; N.S. = not significant, * $P < 0.1$, ** $P < 0.05$, *** $P < 0.01$).

Fig. S4: Evidence that severely impaired time to recovery on 7H11-OADC agar plates is a plausible explanation for why some bacteriostatic antibiotics exhibiting a PAE, and bactericidal compounds, have similar R-CARA results. Wild-type Mtb was exposed to DMSO, PAS or fenamisal at the concentrations shown for 7 days under replicating conditions, and serial dilutions were spread on 7H11-OADC agar plates and incubated for three weeks at 37° C. Colonies were monitored for approximately 2 weeks after counting CFU's to determine if more colonies appeared at later time points. CFU counts in (**a**) were consistent with both PAS and fenamisal acting as bacteriostatic agents. (**b**) There was a dose-dependent decrease in colony size compared to colonies arising from DMSO-treated control cells. The dilutions shown between drug-treated and DMSO vehicle control are not identical and the plates were chosen to illustrate differences in colony size at three weeks. Data shown in (**a**) are means +/- standard deviations of three replicates and error bars are obscured by data points. N=1 experiment.

Fig. S5. Guidelines to determine the CARA-$MBC_{\geq 99}$. The CARA-$MBC_{\geq 99}$ (**a**) is the lowest concentration of drug that results in AB fluorescence remaining at background levels. Although the CARA-$MBC_{\geq 99}$ may appear obvious when graphed (**b**), expanding the Y-axis may reveal a more accurate CARA-$MBC_{\geq 99}$ (**c**). The data are hypothetical.

Fig. S6: Guide to interpreting CARA results. Once accurately determined, the CARA-$MBC_{\geq 99}$ can then be compared with the standard liquid broth MIC to generate a hypothesis as to whether a replicating-active compound is bactericidal (**a**) or bacteriostatic (**b-d**). For some compounds, bacteriostasis requires the identification of a static window (SW) (**d**) that emerges as a > 4-fold shift between the MIC and CARA-$MBC_{\geq 99}$. The example shown in (**e)** demonstrates a candidate non-replicating-active molecule whose NR-CARA suggests activity only at the highest drug concentrations tested; however, this can often be artefactual. In (**f**), a compound may exhibit higher potency by the CARA compared with the MIC_{90} assay, although such cases are rare. The example in (**g**) demonstrates an application of the CARA to identify compounds with pure replicating activity and no non-replicating activity. In general, MIC to CARA-$MBC_{\geq 99}$ differences of 2-fold or lower are likely due to experimental variability. The data are hypothetical.

Table S1. Evidence that TMC207 and PA824 are carried over at growth inhibitory concentrations from liquid broth assays to 7H11-OADC agar plates, and inclusion of activated charcoal in the agar plates can mitigate this effect.

			Fold dilution of test well contents in assay plate prior to spreading on agar plate		
drug	**concentration**	**0.4% (wt/vol) charcoal in agar plate?**	**no dilution**	**1:10**	**1:100**
TMC207	30 μg/mL	no	0, 0, 0	43, 48, 45	5, 10, 6
TMC207	30 μg/mL	yes	too numerous to count	too numerous to count	16, 19, 12
PA824	30 μg/mL	no	0, 2, 0	1, 0, 2	0, 0, 0
PA824	30 μg/mL	yes	25, 20, 24	1, 3, 1	0, 0, 0

Data presented are colony counts from replicating wild-type Mtb exposed to 30 μg/mL TMC207 and PA824 for 7 days. Results of triplicates are separated by commas. 10 μL were removed from a 200 μL assay volume from a liquid MIC assay plate in 96-well format and spread on a 7H11-OADC agar plate with or without 0.4% activated charcoal, directly (undiluted) or diluted 10- or 100-fold in 7H9-ADN medium prior to plating 10 μL. Each aliquot of 10 μL was spread onto 8 mL of 7H11-OADC agar in a Y-style petri plate, effectively diluting any residual drug 800-fold to 0.038 μg/mL. Similar trends, albeit less dramatic, were observed at 10 μg/mL for both drugs (**data not shown**). Representative bacterial counts of one experiment performed 4 times.

Table S2. Physicochemical properties of anti-Mycobacterial molecules. The data in the column, "% removed by 0.4% (wt/vol) activated charcoal", was reproduced from **Table 2** to facilitate data analysis.

Compound	% removed by 0.4% (wt/vol) activated charcoal, 24 hours [f]	MW	log P	HBD	HBA	pKa	Heavy atoms	PSA (Å²)	RB
streptomycin	41.7	582	-7.65	14	19	11.9	40	331.4	9
ethambutol	97.2	204	-0.06	4	4	9.6	14	64.5	9
PA-824	≥ 99.9	359	4.14	0	6	-3.4	25	91.3	6
rifampicin	≥ 99.9	823	2.77	6	14	6.9	59	220.2	5
moxifloxacin	≥ 99.9	401	-0.5	2	7	9.4	29	82.1	4
oxyphenbutazone	≥ 99.9	324	3.83	1	3	4.9	24	60.9	5
ethionamide	≥ 99.9	166	1.33	1	1	5.0	11	38.9	2
isoniazid	≥ 99.9	137	-0.69	2	3	3.4	10	68.0	1
TMC207	≥ 99.9	556	7.13	1	4	8.9	37	45.6	8
linezolid	≥ 99.9	337	0.64	1	5	14.5	24	71.1	4
PAS	≥ 99.9	153	0.83	3	4	3.7	11	83.6	1
fenamisal	≥ 99.9	243	3.22	2	3	9.9	18	72.6	4
nitazoxanide	≥ 99.9	307	2.12	1	5	8.2	21	114.1	5

PSA, polar surface area; HBD, H-bond donor; HBA, H-bond acceptor; RB, rotatable bonds

Figure S1

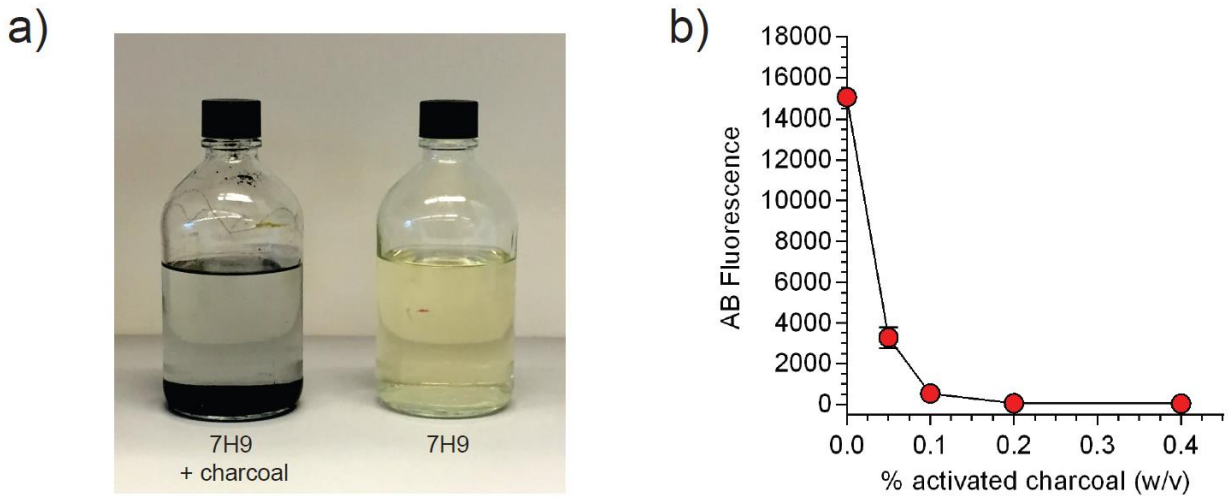

a)
7H9
+ charcoal
7H9
b)
AB Fluorescence
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
0.0
0.1
0.2
0.3
0.4
% activated charcoal (w/v)
c)
7H11-OADC
d)
7H11-OADC-charcoal

Figure S2

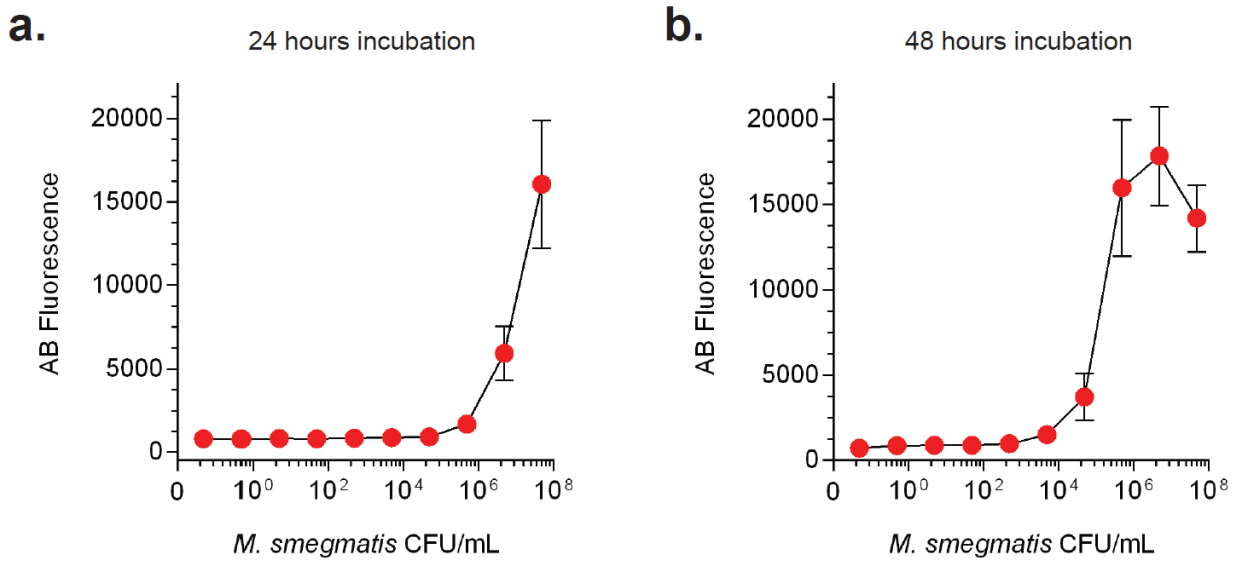

a.
24 hours incubation
AB Fluorescence
20000
15000
10000
5000
0
0
10^0
10^2
10^4
10^6
10^8
M. smegmatis CFU/mL
b.
48 hours incubation
AB Fluorescence
20000
15000
10000
5000
0
0
10^0
10^2
10^4
10^6
10^8
M. smegmatis CFU/mL

Figure S3

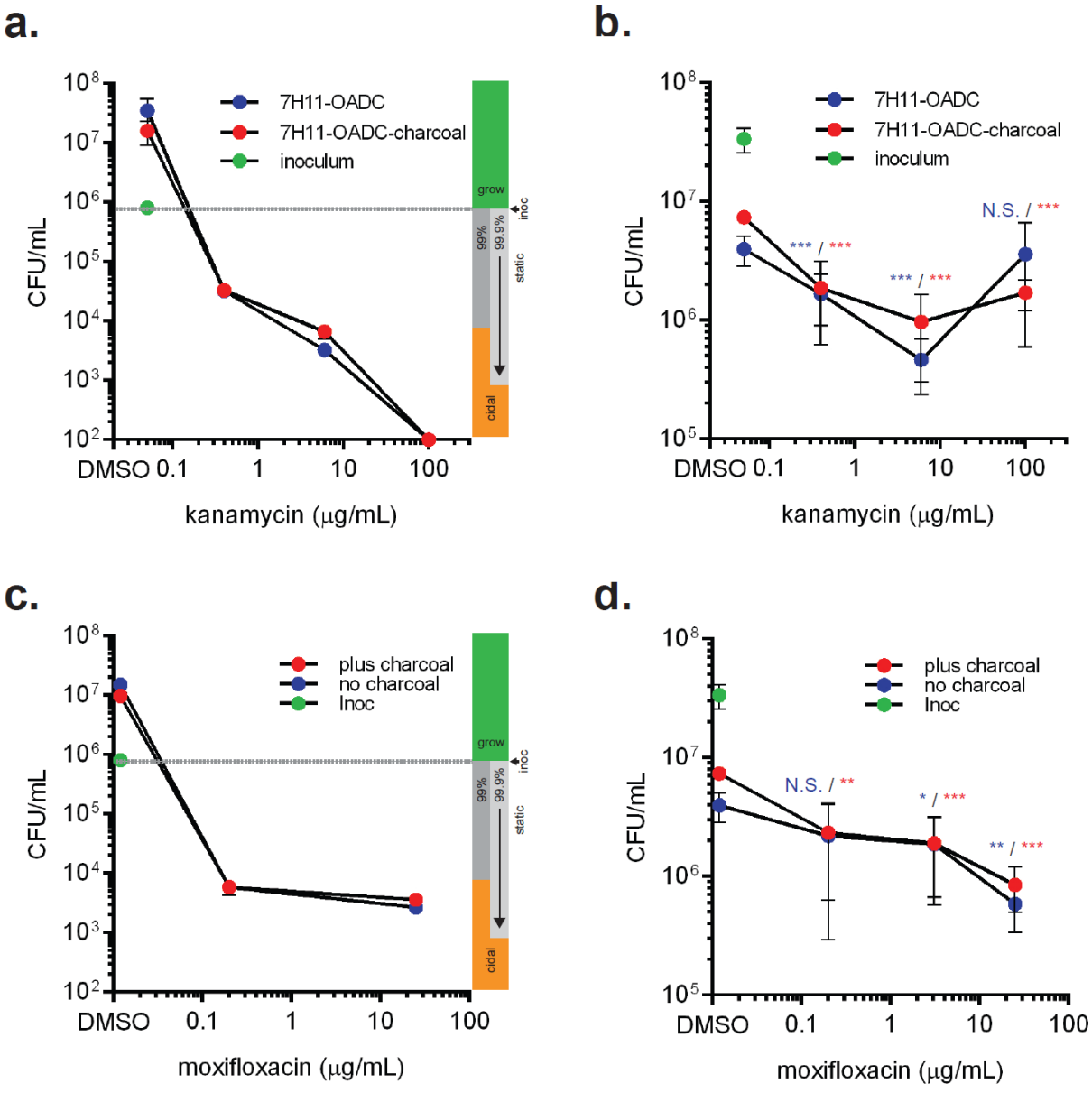
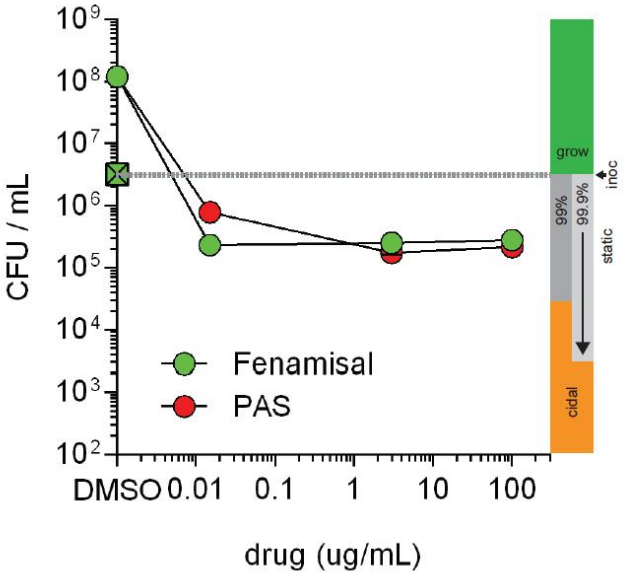

a.
b.
c.
d.
7H11-OADC
7H11-OADC-charcoal
inoculum
plus charcoal
no charcoal
Inoc
CFU/mL
kanamycin (μg/mL)
moxifloxacin (μg/mL)
DMSO
0.1
1
10
100
grow
inoc
99%
99.9%
static
cidal
N.S. / ***
*** / ***
N.S. / **
* / ***
** / ***

Figure S4

a.


CFU / mL
drug (ug/mL)
DMSO
0.01
0.1
1
10
100
Fenamisal
PAS
grow
inoc
99%
99.9%
static
cidal

b.

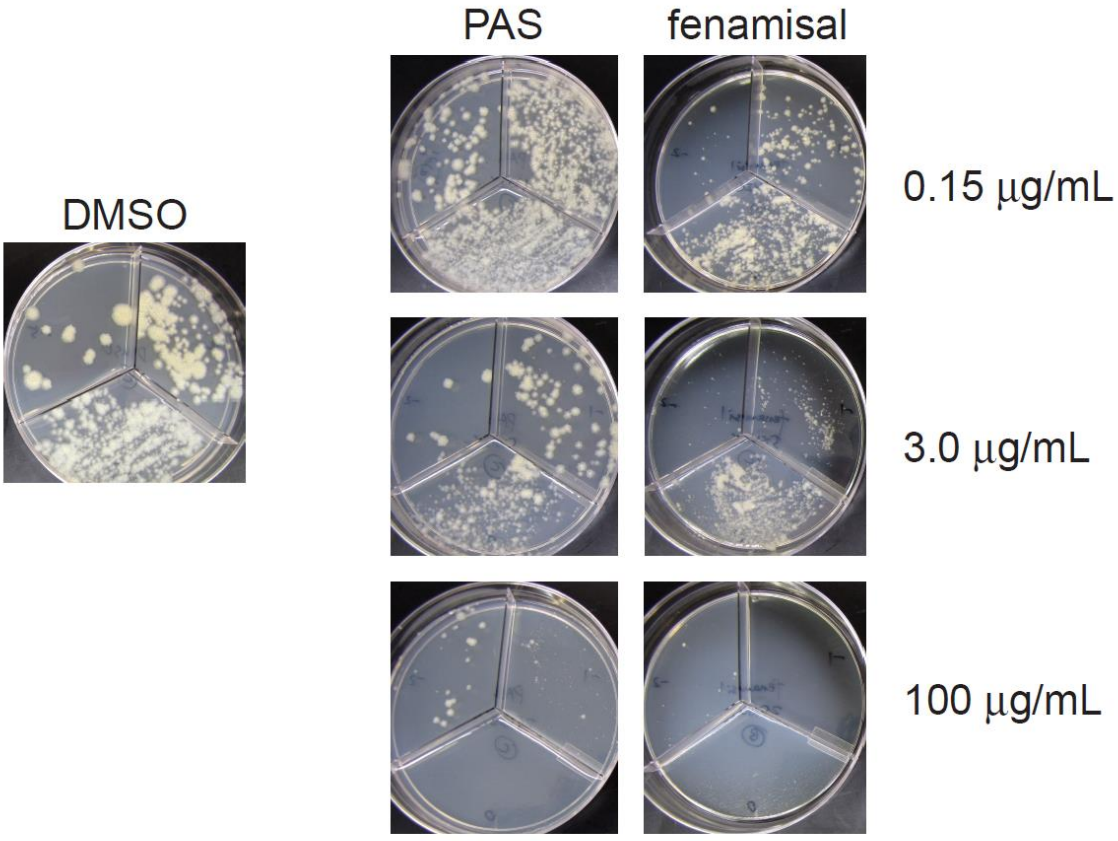

PAS
fenamisal
DMSO
0.15 µg/mL
3.0 µg/mL
100 µg/mL

Figure S5

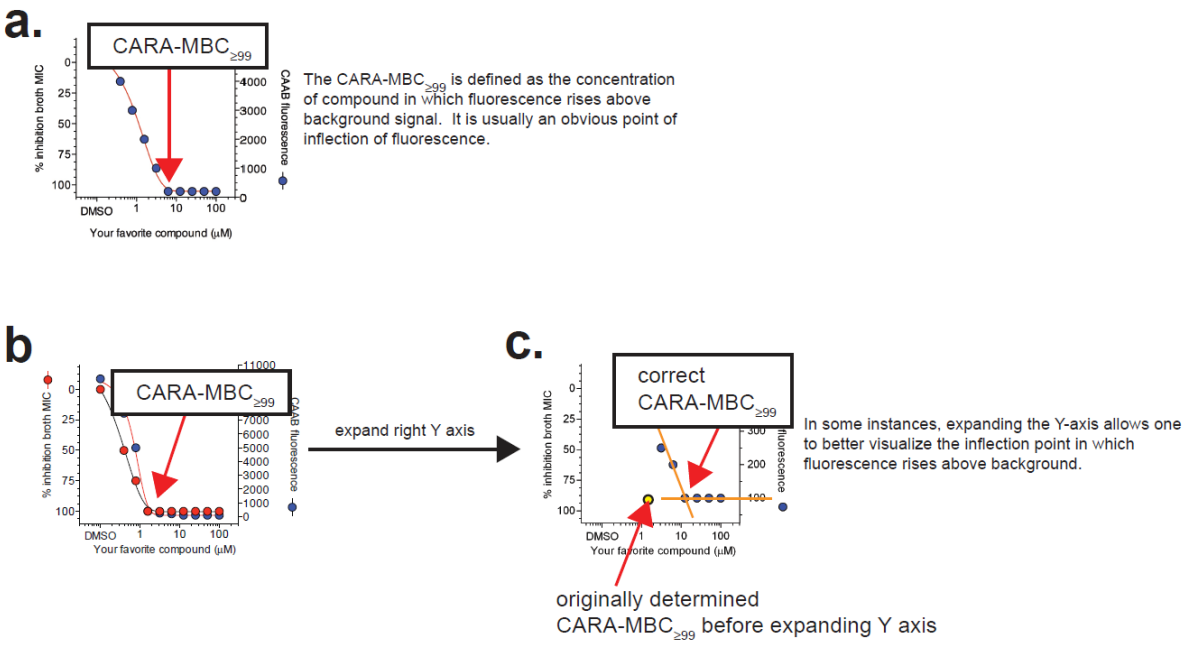
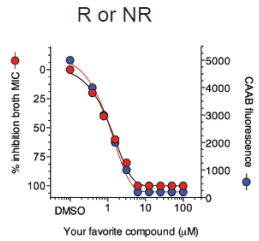

a.
CARA-MBC≥99
% inhibition broth MIC
CAAB fluorescence
DMSO
Your favorite compound (μM)
The CARA-MBC≥99 is defined as the concentration of compound in which fluorescence rises above background signal. It is usually an obvious point of inflection of fluorescence.
b
CARA-MBC≥99
expand right Y axis
c.
correct CARA-MBC≥99
In some instances, expanding the Y-axis allows one to better visualize the inflection point in which fluorescence rises above background.
originally determined CARA-MBC≥99 before expanding Y axis

Figure S6

a.

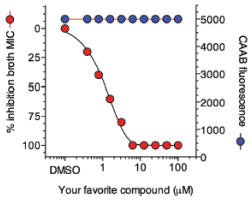


Result: MIC90 = CARA-MBC$_{\geq 99}$

Interpretation: bactericidal

Likely that 2-3 log10 kill will occur at 1X MIC or 1X CARA-MBC$_{\geq 99}$.

b.

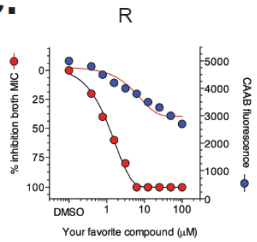


Result 1: MIC90 <<< CARA-MBC$_{\geq 99}$
Result 2: no decrease in fluorescence units in CARA

Interpretation: bacteriostatic, no PAE

This is the easiest to interpret scenario for a bacteriostatic compound. The CARA indicates that no tested concentration of drug leads to an impact on bacterial viability or time to recovery on agar plates.

c.

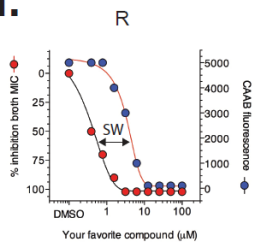


Result 1: MIC90 << CARA-MBC$_{\geq 99}$
Result 2: CARA displays dose-dependent decrease in fluorescence that never reaches background levels.

Interpretation: bacteriostatic, no PAE

In this scenario a bacteriostatic compound still displays no killing in the CARA, even though there is a minor dose-dependent decrease in CARA fluorescence.

d.

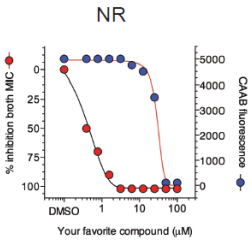


Result 1: MIC90 < CARA-MBC$_{\geq 99}$
Result 2: CARA-MBC$_{\geq 99}$ indicates compound is bactericidal at the highest concentrations tested

Interpretation: "static window" (SW) suggests bacteriostatic; potentially bactericidal at the highest drug concentrations

While the CARA-MBC$_{\geq 99}$ indicates that the compound is bactericidal at higher drug concentrations, there is a significant shift between the MIC90 and CARA-MBC$_{\geq 99}$ (>4-fold in this example). We have observed this "static window" for bacteriostatic drugs such as linezolid. It is unexplained why CARA data suggests the compound is bactericidal at the highest drug concentrations. One possibility is that these drug concentrations lead to a post-antibiotic effect that restricts the time it takes surviving cells to recovery on agar plates as visible colonies, yet fails to decrease the CFU numbers.

e.

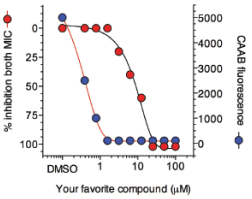


Result 1: NR-MIC << NR-CARA-MBC$_{\geq 99}$
Result 2: potent R-active by MIC and CARA (not shown)
Result 3: CARA-MBC$_{\geq 99}$ fluorescence is at background levels only at the highest concentrations tested and not dose-dependent

Interpretations: 1) NR-inactive; 2) failure of activated charcoal to effectively remove highest concentrations of drug

The absence of a dose-dependent decrease in Alamar blue fluorescence at the majority of drug concentrations tested, coupled with a profound decrease in fluorescence only at the highest 1-2 drug concentrations, suggests this compound is not bactericidal under NR conditions. The impact on CARA fluorescence is likely due to compound carry-over. Note the concept of a bacteriostatic drug does not apply to NR conditions.

f.

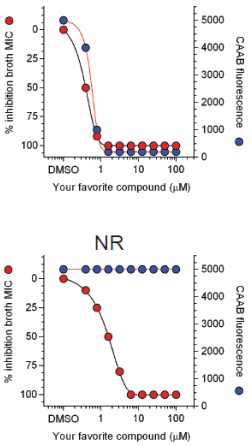


Result: MIC90 > CARA-MBC$_{\geq 99}$

Interpretation: unknown

Uncommon result in which the CARA suggests a compound is highly bactericidal, yet the liquid broth MIC suggests the compound is not very potent. Compounds that interfere with cell shape (for example, cells filamenting or lysing) might lead to artefacts when measuring bacterial biomass with optical density as a readout. Alternatively, compound precipitation could also lead to optical density artefacts.

g.



Result 1: R MIC90 = R CARA-MBC$_{\geq 99}$
Result 2: NR MIC90 > R MIC90
Result 3: no decrease in fluorescence units in NR-CARA

Interpretation: R bactericidal, NR inactive

This example demonstrates a potent R-active that has carried over from the liquid NR MIC90 assay to the replicating outgrowth phase. Such a compound appears to be NR active. The NR-CARA, by removing carry-over drug, demonstrates that this compound is completely inactive in the NR conditions.