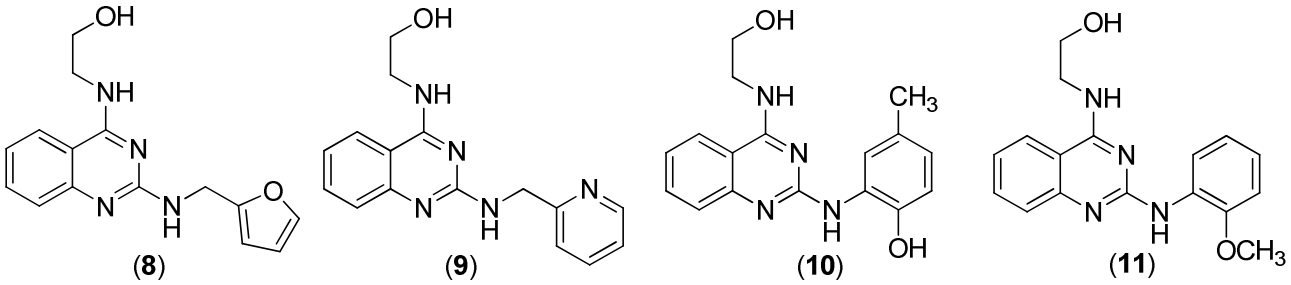

Fig. S1. Compounds that partially inhibited motility. These compounds were tested for inhibition of motility in swarm agar at a concentration of 10 μg/ mL.

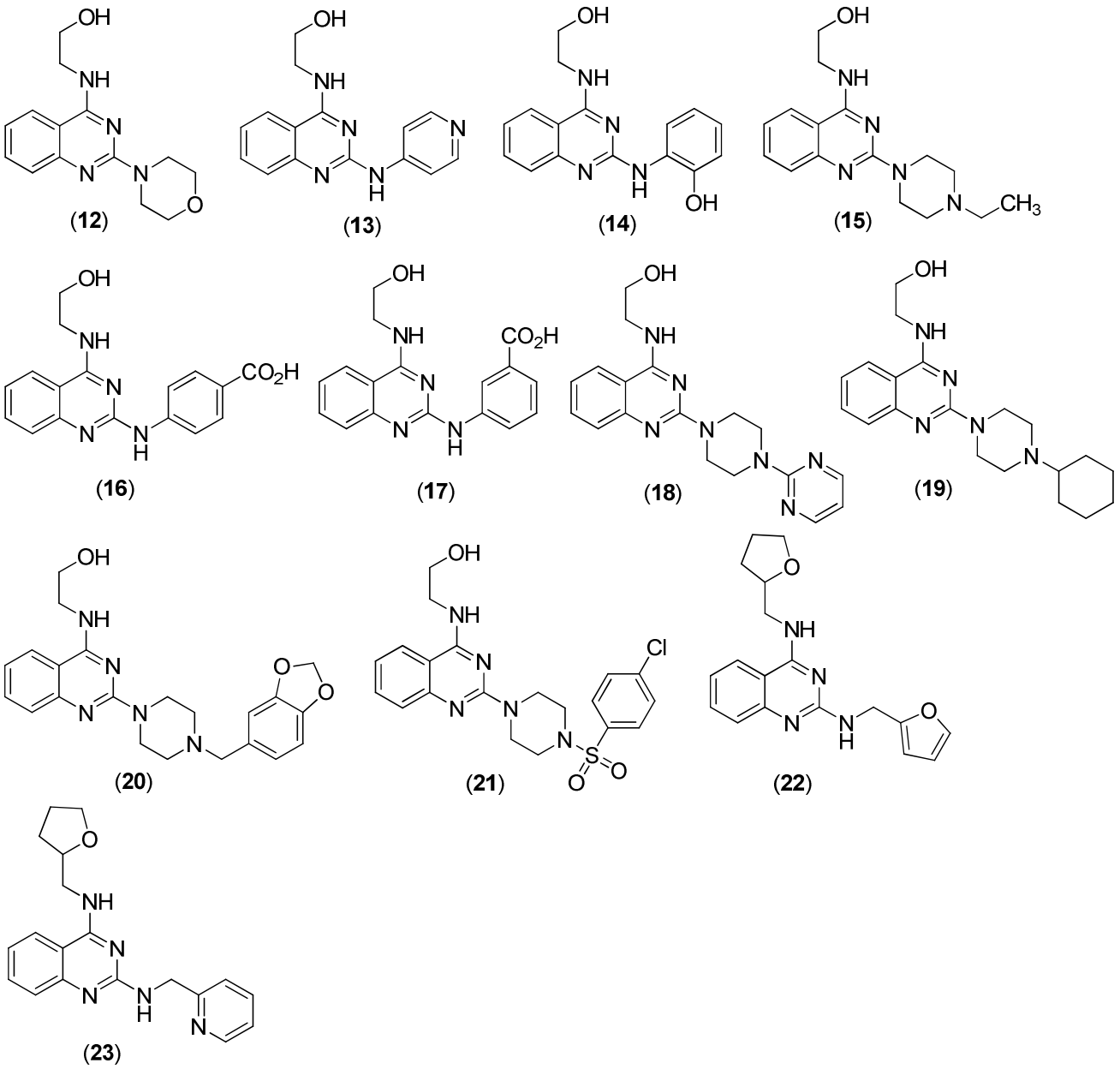

Fig. S2. Compounds showing no effect on motility. These compounds were tested for inhibition of motility in swarm agar at a concentration of 10 μg/ mL

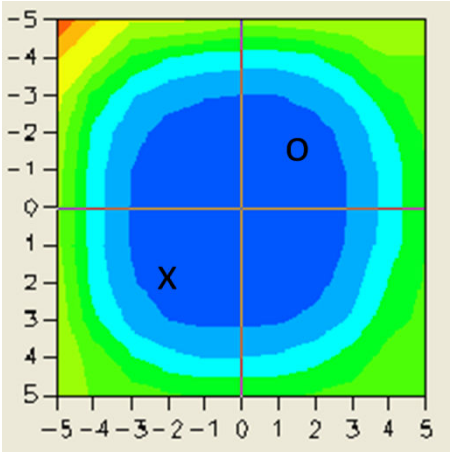

Fig. S3. **Schematic of motility assay.** Visualization of one 384 well. By offsetting the read location from the inoculation site, non-motile bacterial colonies did not interfere with the absorbance read. Crosshair marks well center, X is the inoculum site, and O is the read site.

SOFT AGAR MOTILITY HTS ASSAY METHOD

Assay Plates. The assay was conducted in 384-well clear bottom microtiter plates. A Wellmate non-contact dispenser (Matrix/Thermo) was used to add 50 μL of LB containing 0.3% agar to the assay plates. Agar was maintained at 40ºC during plating to prevent solidification. Plates were sealed immediately after agar addition and maintained at room temperature for at least 4 h to allow agar to solidify. Drug delivery was done with a Labcyte Echo 550 liquid handler which transferred 50-nL of test compound into the test well of the assay plate. Compounds dispensed to the top of the soft agar were allowed to diffuse for 2-4 h at room temperature.

Bacterial inoculation. An Echo 555 liquid handler (Labcyte) was used to transfer 2.5 nL of bacterial culture, containing approximately 20 CFU of strain C7258, to the soft agar assay plate. The 2.5-nL drop of bacterial culture was delivered to the bottom left corner of the well which was accomplished by adjusting the well offset for the destination plate in the labware profile on the Echo 555. Plates were incubated at 30ºC with high humidity for 14 h.

Endpoint read. Plates were read using absorbance at 615 nm on an Envision multimode plate reader (Perkin Elmer). The read location in the well was offset diagonally to allow maximum distance between the inoculation site and the read location. This was accomplished with the plate dimension optimization tool in the Envision software. This strategy is illustrated in the **Fig. S3**. The primary assay readout was absorbance at 615 nm to monitor bacterial growth and motility from the inoculation site to the read location. A high absorbance indicates no inhibition of motility. A low absorbance indicates either inhibition of motility or antimicrobial activity. To differentiate between non-motile and non-viable, 5 μL 100 % alamarBlue was added to each well following the absorbance read. Plates were incubated for an additional 60 min at 30ºC and then fluorescence intensity was determined by reading with an excitation wavelength of 535 nm and an emission wavelength of 590 nm on the Envision. Non-motile viable bacteria convert the alamarBlue which produces an increase in fluorescence signal, while non-viable cells do not convert the alamarBlue and the fluorescence signal remained low. This allowed the discrimination between antimicrobial compounds and motility inhibitors. **Table S1** contains

absorbance and fluorescence data for motile and non-motile bacteria with and without tetracycline, a known bactericidal compound, to illustrate how the two reads are used to define anti-motility compounds. We show that the absorbance for motile bacteria is high while non-viable (+ Tet) or non-motile (Mot^-) are low. Viability determined by addition of alamarBlue clearly differentiates between non-viable (low fluorescence) and viable (high fluorescence) for both motile and non-motile strains.

Data analysis. Thirty two wells: 24 containing tetracycline (antimicrobial) (5 μg/mL) and 8 containing phenamil (anti-motility) (0.1 mM) were included on each assay plate. Compound data was normalized to plate controls and reported as % inhibition which was calculated using the following formula for either absorbance or fluorescence endpoints: % inhibition = 100*[1-(Compound value – positive control)/(negative control – positive control) where the positive control is tetracycline and the negative control is bacteria plus DMSO. The normalized % inhibition was plotted against the tested concentrations. The IC_{50} values were calculated using XLfit formula 205, a 4 parameter Levenburg-Marquardt algorithm with maximum and minimum limits set at 100 and 0, respectively.

Table S1. Absorbance and fluorescence values for motile, non-motile and non-viable controls

	Strain C7258	**C7258 + Tet**	**C7258Mot$^-$**	**C7258Mot$^-$ + Tet**
Absorbance	1.25	0.25	0.29	0.24
Fluorescence	8,082,494	272,548	9,869,575	255,445