

RESEARCH ARTICLE

Bilayer Effects of Antimalarial Compounds

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Abstract

Because of the perpetual development of resistance to current therapies for malaria, the Medicines for Malaria Venture developed the Malaria Box to facilitate the drug development process. We tested the 80 most potent compounds from the box for bilayer-mediated effects on membrane protein conformational changes (a measure of likely toxicity) in a gramicidin-based stopped flow fluorescence assay. Among the Malaria Box compounds tested, four compounds altered membrane properties ($p < 0.05$); MMV007384 stood out as a potent bilayer-perturbing compound that is toxic in many cell-based assays, suggesting that testing for membrane perturbation could help identify toxic compounds. In any case, MMV007384 should be approached with caution, if at all.

Introduction

Malaria continues to be a dangerous illness, causing 584,000 fatalities in impoverished, tropical regions in 2010 [1]. The pathogenesis of the disease and the perpetual emergence of resistance necessitates combination therapy and the need for continuous addition of new drugs to the pipeline [2]. Yet, despite continued efforts to develop new drugs, there currently are no approved drugs for malaria that do not have documented resistance [3].

This is a serious problem because, in the case of neglected tropical diseases (NTDs) that predominantly infect people in developing nations, the cost of developing a drug is a critical limiting factor. It thus becomes important to develop tools to identify likely failures early in development, and thereby decrease the cost of drug discovery and development. A key concern in this context is safety, given the populations that are most at risk of succumbing to malaria and the need for widespread administration in an asymptomatic population, as recommended by the current eradication agenda [4].

To facilitate the development of novel drug candidates and biological probes for treating and studying malaria, the Medicines for Malaria Venture (MMV, <http://www.mmv.org>) began distributing the Malaria Box in 2012 [5]. The compounds chosen for inclusion in the Malaria Box were selected based on collective phenotypic screening at GlaxoSmithKline, Novartis, and St. Jude Children's Research Hospital. 30,000 unique structures displayed phenotypic hits at

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submicromolar concentrations, and the MMV included a limited diverse set that had undergone principal component analysis, physicochemical profiling, and determination of commercial availability. This final product was split into two categories of drug-like and probe-like using Rule-of-Five compliance [6] and substructure filters to eliminate molecules that for a variety of reasons are likely to be problematic [7] including PAINS (pan assay interference compounds) [8].

Due to the predominantly intracellular nature of the malaria life cycle, successful drugs will need to cross cell membranes—and, unless the compound can enter cells by a protein-catalyzed process [9], it will need to traverse the membrane's lipid bilayer [10]. Such uncatalyzed solute movement across lipid bilayers involves partitioning into the bilayer and diffusion through the hydrophobic core (the solubility-diffusion mechanism [11]). The joint requirements, that successful antimalarials need to be both sufficiently water-soluble to distribute throughout the body and sufficiently lipid-soluble to cross cell membranes to reach their intracellular targets, entail that antimalarials, like many other drugs, are amphiphiles—as would be expected from Lipinski's Rule of Five [6, 12].

It long has been appreciated that drugs may alter lipid bilayer properties [13], and drug-induced in lipid bilayer structure and dynamics have been demonstrated using a variety of spectroscopic methods in the case of antidepressants [14, 15] phytochemicals [16, 17] and antimicrobial peptides [18, 19]—and many biologically active amphiphiles alter lipid bilayer properties and integral membrane protein function at similar concentrations [20–25]. This bilayer regulation of membrane protein function arises because membrane protein function depends on conformational transitions that involve the proteins' bilayer-spanning domains—leading to alterations in lipid packing adjacent to the proteins of interest. Such bilayer deformations incur an energetic cost, the bilayer deformation energy (ΔG_{def}) such that the free energy difference between different protein conformations (I and II) includes a contribution from the difference in bilayer deformation energy associated with the two protein conformations: $\Delta G_{\text{total}}^{\text{I} \rightarrow \text{II}} = \Delta G_{\text{protein}}^{\text{I} \rightarrow \text{II}} + \Delta G_{\text{bilayer}}^{\text{I} \rightarrow \text{II}}$, where $\Delta G_{\text{protein}}^{\text{I} \rightarrow \text{II}}$ denotes the energetic contribution from rearrangements within the protein and $\Delta G_{\text{bilayer}}^{\text{I} \rightarrow \text{II}}$ ($= \Delta G_{\text{def}}^{\text{II}} - \Delta G_{\text{def}}^{\text{I}}$) the contribution from the bilayer (the different lipid packing associated with the different conformations).

$\Delta G_{\text{def}}^{\text{I}}$ and $\Delta G_{\text{def}}^{\text{II}}$, and thus $\Delta G_{\text{bilayer}}^{\text{I} \rightarrow \text{II}}$, vary as function of the bilayer physical properties [26, 27], meaning that changes in bilayer lipid composition, such as the addition or removal of cholesterol or the partitioning of amphiphiles into the lipid bilayer/solution interface (and the ensuing changes in bilayer properties), will alter membrane protein function. Experimental maneuvers that increase bilayer stiffness, such the addition of cholesterol, thus will alter membrane protein function [28], with the direction of the change determined by the changes in $\Delta G_{\text{def}}^{\text{II}}$ relative to the changes in $\Delta G_{\text{def}}^{\text{I}}$. Amphiphile-induced changes in lipid bilayer properties similarly have been observed with different classes of molecules: detergents and lipid metabolites including poly-unsaturated fatty acids [22, 29–31], phytochemicals [16, 20, 23, 25], and drugs [14, 24], see also [32].

Membrane effects have been correlated with toxicity and screening for membrane effects have been proposed as a cost and time effective way to measure toxicity, using lipid vesicle-based systems [33], and drugs that are potent modifiers of membrane (lipid bilayer) properties previously have been shown to have an increased risk of failing as safe drugs [24]. We therefore decided to explore whether antimalarial drug candidates cause membrane effects, and, if so, whether these effects are large enough to be of concern—whether the drugs are likely to produce indiscriminate changes in membrane protein function that would be expected to cause undesired effects.

Screening drug candidates for bilayer effects thus might reduce the resources spent on drug development by providing information about small molecule promiscuity at an early stage in development. Using the eighty most potent Malaria Box compounds (40 drug-like and 40-probe-like), we screened for membrane (off-target) effects using a gramicidin-based assay that has been validated to predict membrane-mediated effects on integral membrane proteins [21, 24, 25, 34, 35].

Gramicidin channels form by transmembrane dimerization of non-conducting monomers, and channel formation leads to a local bilayer thinning with an energetic cost that varies with (drug-induced) changes in lipid bilayer properties. Changes in the equilibrium distribution between non-conducting monomers and conducting dimers can be quantified using electrophysiological [32] and fluorescence [36] methods. We used a gramicidin-based fluorescence assay (GBFA) that employs fluorophore-loaded large unilamellar vesicles (LUVs) doped with gramicidin and a gramicidin channel-permeable heavy ion quencher (Tl^+), where changes in the gramicidin monomer \leftrightarrow dimer equilibrium are monitored as changes in the time course of fluorescence quenching. The assay has previously been used to screen a library of compounds that inhibited acid-base homeostasis in *M. tb.* [37], where over half of the candidate hits identified in a whole screen (for inhibitors of pH homeostasis) altered bilayer properties. Would the Malaria Box compounds have a similar proportion of compounds that modulate bilayer properties?

Materials and Methods

LUVs were prepared from 1,2-dierucoyl-*sn*-glycero-3-phosphocholine in chloroform (Avanti Polar Lipids, Alabaster, AL) and gramicidin (Sigma Chemical Co, St. Louis, MO) in methanol (lipid:gramicidin molar ratio 2000:1), and loaded with the fluorophore 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) disodium salt (Invitrogen, Eugene, OR) using a freeze-thaw and extrusion method, as described previously [36, 38, 39]. The lipids and gramicidin were dissolved in chloroform:methanol, the solvents were removed by dried under nitrogen until the lipid:gramicidin mixture appeared to be dry and followed by incubation in a desiccator overnight. The dried lipid:gramicidin film was rehydrated in 100 mM $NaNO_3$, 25 mM ANTS, and 10 mM HEPES (pH 7.0) at room temperature for 3–4 hours or overnight. The mixture was sonicated at low power for 1 min, subjected to 5 freeze–thaw cycles, and extruded 21 times, at room temperature, using an Avanti mini-extruder or Lipex 10 mL extruder and a 0.1 μ m polycarbonate membrane filter. Extravesicular ANTS was removed using a PD-10 Desalting column (GE Healthcare, Piscataway, NJ), and the ANTS-LUV stock solution was stored at 12°C, in the dark, for a maximum of 10 days. The ANTS-LUV stock solution was diluted 1:20 with 140 mM $NaNO_3$ plus 10 mM HEPES (pH 7.0) after incubating for 24 h at 12°C in the dark.

All molecules were provided by the MMV in the 80 compound Malaria Box; they were diluted in DMSO and stored at -40°C.

The GBFA was performed as described previously [39]. The time course of fluorescence quenching was measured at 25°C using an SX.20 Stopped-Flow Spectrometer (Applied Photophysics, Leatherhead, UK), with a dead time \sim 1.2 ms. The excitation was at 352 nm and the fluorescence was recorded above 455 nm using a high-pass filter and a sampling rate of 5,000 points/s. For each experimental condition, at least 5 repeated mixing trials were measured, from 1 or more vesicle preparations. Because of the inevitable variation in LUV sizes, the time course of fluorescence quenching was normalized to the average fluorescence without quencher for that sample, and the first 2–100 ms was fitted with a stretched exponential [36, 40]. The initial influx rate was estimated from the rate of the stretched exponential at 2 ms and normalized to the influx rate in the presence of vehicle only. Unpaired t-tests were performed

using PRISM (GraphPad Software, Inc., La Jolla, CA). *P* values less than 0.05 were considered statistically significant.

Results

Fig 1 shows results obtained when fluorophore-loaded, gramicidin-doped LUVs are mixed with a gramicidin channel-permeant quencher (TI^+) in a stopped-flow spectrofluorometer. The fluorescence decrease reflects the influx of TI^+ into the vesicle, which quenches the fluorophore (ANTS) fluorescence. In the absence of gramicidin, there is no change in fluorescence. The fluorescence quench rate was accelerated in the presence of a bilayer-modifying amphiphile, such as MMV007384, which is 5,5'-Methylenebis [2- (4-methoxyphenyl)-1H-benzimidazole] [5]. Using quench rates determined from the fluorescence traces (similar to those depicted in Fig 1), dose-response curves (1–10 μM) were constructed for each compound; the results are summarized in S1 Fig. To visualize whether the Malaria Box compounds alter lipid bilayer properties, Fig 2 shows the normalized rates (relative to control, in the absence of the compound in question) for each compound at 5 μM (the Malaria Box compounds' EC_{50} vary between ~ 10 nM and 3 μM [5], and MMV recommends testing at 1 μM ; we chose the results obtained at 5 μM to visualize whether or not the compounds altered the fluorescence quench rate). Four of the 80 Malaria Box compounds alter lipid bilayer properties as evident by increased quench rates as compared to control (the chemical structures of these four compounds are depicted in S2 Fig).

Whether or not the Malaria Box compounds altered lipid bilayer properties, they did not compromise the lipid bilayer barrier properties—there was no evidence for leakage of ANTS out of the LUVs during the 10 min incubation with 5 or 10 μM of the Malaria Box compounds (which would be observed as an instantaneous decrease in fluorescence when the LUVs were

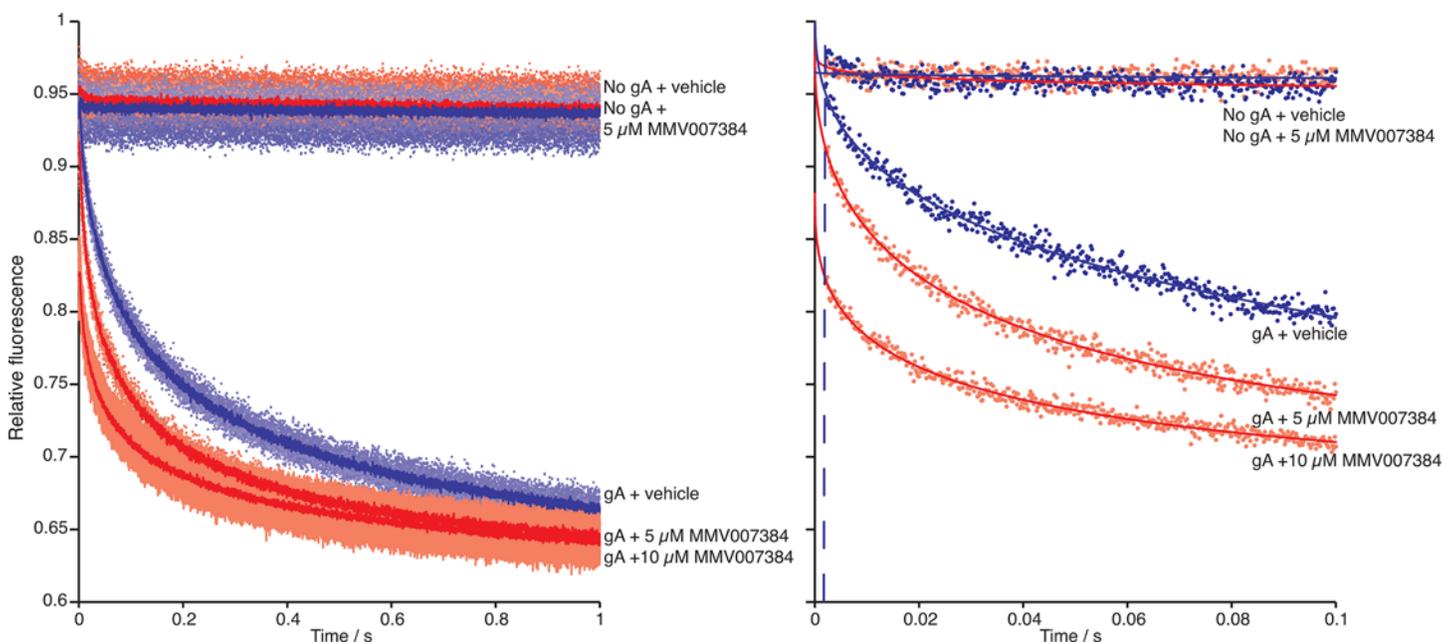


Fig 1. MMV007384 is a bilayer-modifying compound. Fluorescence quench traces showing the effects of 5,5'-Methylenebis [2- (4-methoxyphenyl)-1H-benzimidazole] or MMV007384 [5] in the gramicidin-based fluorescence assay (GBFA). The panel left shows the results over 1 s. In the presence of 5 or 10 μM of the Malaria Box compound, the quench rate normalized to control (in the absence of the compound) increased to 1.8 (q_5) and 2 (q_{10}), respectively. The difference is statistically significant ($p < .0001$). The right panel shows the average trace for a single run at higher time resolution.

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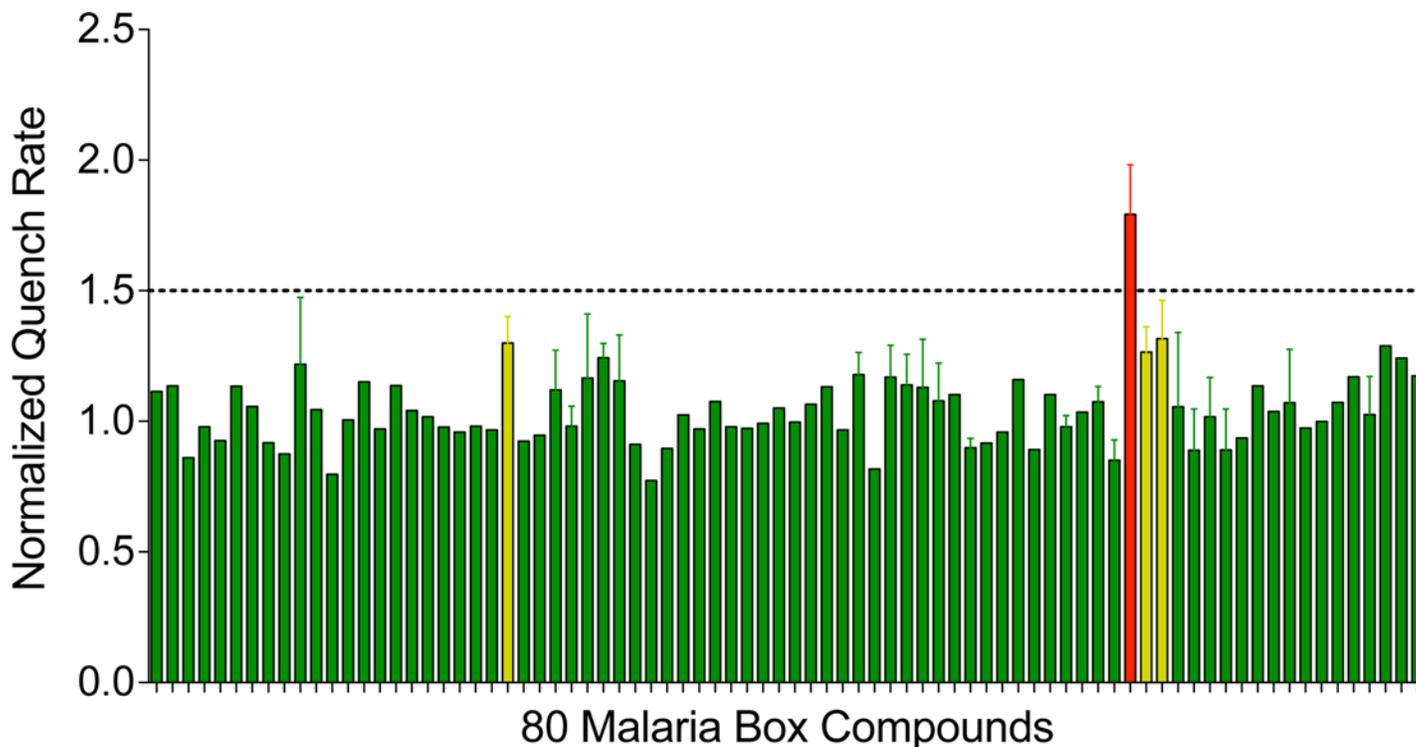


Fig 2. Four Malaria Box compounds have statistically significant bilayer effects. 80 Malaria Box compounds were tested at 5 μ M, the order of the compounds is A2 . . . A11, B2 . . . B11, etc. The four Malaria Box compounds that perturb the bilayer are MMV007384 (red bar, $q_5 = 1.79$, $p < 0.01$), and from L to R (amber bars): N-{4-[(4-methyl-1-piperidiny)methyl]phenyl}-1H-pyrrolo[3,2-h]quinoline-2-carboxamide, MMV020549, $q_5 = 1.3$, $p < 0.05$); N-{4-[(4-ethyl-1-piperaziny)methyl]phenyl}-1H-pyrrolo[3,2-h]quinoline-2-carboxamide, MMV020548, $q_5 = 1.27$, $p < .05$); and 2-[[1-(2,4-dimethoxyphenyl)-1H-imidazol-2-yl]sulfanyl]-N-(2,5-dimethylphenyl)acetamide MMV019258, $q_5 = 1.32$, $p < .05$). Error bars depict S.E.M. Unpaired, two-tailed t-tests were performed in comparison to control.

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mixed with the Tl^+ -containing quench solution). Nor did the Malaria Box compounds (other than the four in Fig 2) increase the rate of Tl^+ influx, in the absence or presence of gramicidin, meaning that they did not produce leaks in the LUV membrane.

The most potent of the four membrane-active Malaria Box compounds, MMV007384, is classified by MMV as a probe-like compound and has a bilayer-modifying potency that is correlated with toxicity to cells in culture [41]. In fact, MMV007384 is known to have a high IFI Promiscuity Index (S3 Fig), which is a measure of activity in high-throughput toxicity assays performed at Novartis-GNF or GSK, as well as low IC_{50} s for drug-resistant strains of *P. falciparum* and several NTDs such as *T. brucei rhodesiense*, *L. infantum*, *T. brucei*, *T. cruzi*, *M. tuberculosis* and cytotoxicity against MRC-5 human fibroblasts and the human hepatoma cell line Huh7 (S4 Fig).

Many drug-screening assays use physicochemical parameters and substructure filters, e.g. calculated octanol/water partition coefficients (cLogP) [42], Lipinski's "Rule of Five" [6], or the Rapid Elimination of Swill (REOS) [7], the screen for Pan Assay Interference Compounds (PAINS) [8], or the Quantitative Estimate of Drug-likeness (QED) [43], to evaluate/predict a compound's drug-likeness, bioavailability or toxicity. We analyzed the Malaria Box compounds according to physicochemical parameters like cLogP (Fig 3), Lipinski's Rule of Five (five of the 80 Malaria Box compounds violated two of Lipinski's guidelines) and QED (Fig 4, S5 Fig), as calculated using Pipeline Pilot (Accelrys Inc., San Diego, CA).

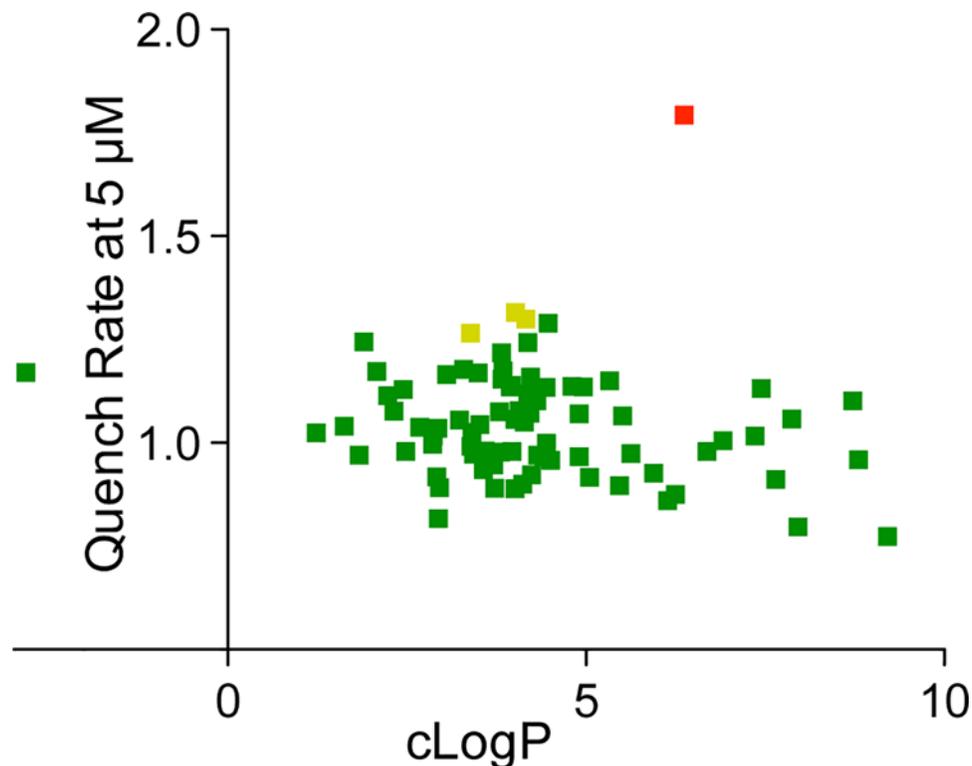


Fig 3. The Malaria Box compounds' estimated hydrophobicity (cLogP) is not correlated with lipid bilayer effects. Plot of the quench rate (relative to control) vs. cLogP, which was calculated using the ALOGP algorithm [44] as implemented by Collaborative Drug Discovery, Inc. (<http://collaborativedrug.com>, Burlingame, CA). MMV007384 (red dot cLogP = 6.37); from L to R (amber dots): MMV020548, cLogP = 3.39; MMV020549, cLogP = 4.16; and MMV019258, cLogP = 4.01.

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The Malaria Box compounds' cLogP values are distributed between -2.5 and 10, with the drug-like compounds having cLogP values between 1 and 5 (in accordance with Lipinski's Rule of Five; by design, most Malaria Box drug-like compounds fail none of the criteria in the Rule of Five). When evaluating the Malaria Box compounds using the quantitative estimate of drug-likeness (QED) measure [43] there is clustering around "attractive" QED (high drug-likeness) values (Fig 4). Overall, there is little correlation between bilayer-perturbing effects and any of the "standard" measures of drug-likeness, suggesting that the GBFA is a novel tool to be considered when evaluating the likely toxicity of drug candidates. Employing the GBFA is concordant with the "quick win, fast fail" drug development paradigm, which proposes that technical uncertainty be decreased before expensive later development stages are embarked upon [45].

Discussion

In this study, we explored whether the compounds provided in the Malaria Box have significant bilayer-modifying effects. We find that, as might have been predicted *a priori* based on the criteria used to select the Malaria Box compounds, that only 5% of the compounds have statistically significant effects on lipid bilayer properties. Only one of these, MMV007384, alters the fluorescence quench more than 50%, suggesting that it might alter membrane protein function and thereby cause unwanted effects. Indeed, MMV007384 is categorized as a probe-like compound with considerable toxicity in cellular studies (<https://www.ebi.ac.uk/chembl/>),

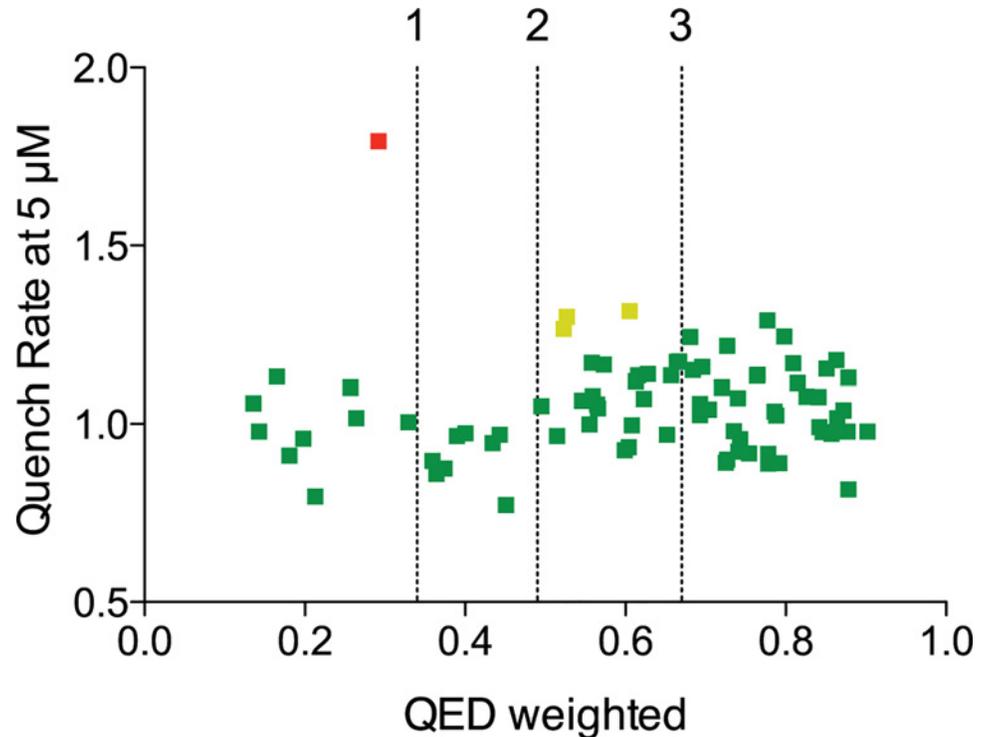


Fig 4. QED does not correlate with bilayer-mediated off-target effects. Plot of the quench rate (relative to control) vs. the weighted QED [43]. There is some clustering of the Malaria Box compounds toward the “attractive” QED value. MMV007384 (red dot, weighted QED = .292) is an unattractive complex compound. **From L to R** (amber dots): MMV020548 (weighted QED = .523) and MMV020548 (weighted QED = .527) are unattractive complex compounds; and MMV019258 (weighted QED = .605) is at the cusp of attractive and unattractive simple compounds. Vertical dotted lines represent reported averages of, from L to R: unattractive (complex); unattractive (simple); and attractive QED values. QED was calculated using Pipeline Pilot (Accelrys Inc., San Diego, CA). (Weighted QED did not differ substantially from unweighted QED, not shown).

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suggesting that studies on the bilayer-modifying effect of drug candidates may provide additional information on their safety (as well as identifying possible mechanisms for any toxicity).

It is in this context important that antimalarials need to cross at least one cell membrane in order to exert their action. They can do so via protein-catalyzed transfer [9, 10] or by the solubility-diffusion mechanism [11]; in the latter case, the drugs will partition into the lipid bilayer/solution interface and thereby alter lipid bilayer properties. The question thus becomes whether the changes in bilayer properties are of sufficient magnitude to be relevant because different classes of drugs have different bilayer-perturbing propensities, e.g., [24, 35]. A previous screen for compounds that disrupt the pH homeostasis in *M. tb.* [37], as well as a recent study on phytochemicals [25] shows that many biologically active compounds have the capacity to be bilayer-perturbing at the concentrations where they alter membrane protein function, and membrane-active compounds such as capsaicin and curcumin alters lipid bilayer structure and dynamics as visualized using nuclear magnetic resonance [16, 17]. These results, together with previous studies on the bilayer-perturbing effects of biologically active compounds [20–22, 24, 25, 31, 46], further demonstrate that bilayer-modifying effects are generalizable, in the sense that the changes in gramicidin channel function are similar in bilayers of different composition [20, 22, 24, 31, 46], including cell membranes [47]—and that one can predict amphiphile-induced changes in

integral membrane protein function from the changes in gramicidin channel function in the synthetic systems we have developed [21, 24, 25].

The release of the Malaria Box has stimulated studies on the compounds provided in the box, leading to a number of discoveries. In studies on *P. falciparum*: Fletcher and Avery [48] showed that 12 Malaria Box compounds are potential inhibitors of the Coenzyme A pathway; Hain et al. [49] identified a target (Atg8-Atg3 protein-protein interaction) for six Malaria Box compounds in the parasite's blood and liver stages; Lucantoni et al. [50] found 64 gametocidal compounds amongst the Malaria Box compounds; and Paiardini et al. [51] found two compounds active against neutral aminopeptidases M1 and M17. In studies on other parasites: Boyom et al. [52] showed that seven Malaria Box compounds are potent inhibitors of *Toxoplasma gondii*, and two are potent inhibitors of *Entamoeba histolytica*; Alemán Resto et al. [53] identified three compounds that may target the oyster Dermo Disease caused by *Perkinsus marinus*; Njuguna et al. [54] identified two compounds that appear to target the tick *Theileria parva*, which causes East Coast Fever in cattle; and Kaiser et al. [55] found nine promising *in vitro* candidates that inhibited growth of *Trypanosoma* and *Leishmania* species.

Three of the compounds identified in the above studies (MMV007384, MMV020549 and MMV020548) are among those we have shown to perturb the lipid bilayer. Compounds MMV007384 and MMV020548 are potent gametocidal agents [50], and MMV020549 has activity against *E. histolytica* [52]. These compounds should be approached with caution given the likelihood of membrane effects, which is likely to produce undesired toxicity in long-term therapy (or prophylaxis among asymptomatic patients), a concern that is magnified by the need to use concentrations that are ten times higher than EC_{50} in order to inhibit a target by 90%.

Given the results on compounds that disrupt pH homeostasis in *M. tb.* [37], it is surprising that only four of the compounds had a significant effect on membrane properties. We attribute the difference between the results in the current study and those of Darby et al. to the different stages where the two groups of compounds are in the discovery process. Compared to the tuberculosis compounds, the proportion of Malaria Box compounds that are membrane active is much less, most likely because the stage of the discovery process for the Malaria Box compounds is more advanced. We are pleased to report that 76 of the 80 most potent Malaria Box compounds have no discernible bilayer effects at concentrations up to 10 μ M, meaning that they are unlikely to have side effects associated therewith.

Supporting Information

S1 Fig. Dose-response curves for the Malaria Box compounds' bilayer-modifying propensities using the GBFA. Eighty molecules were tested; the results are separated into eight graphs, each depicting results for 10 molecules, which are identified by their well positions in the Malaria Box plate. The interrupted lines at 1.5 on the y-axes correspond a cut-off for bilayer-mediated effects when interpreting the GBFA results. The compounds identities at the indicated plate positions can be found in Table S1 from Spangenberg et al. [5]. (MMV020549 is C4; MMV007384 is G3; MMV020548 is G4; and MMV019258 is G5). (EPS)

S2 Fig. Chemical Structures of the bilayer-perturbing Malaria Box molecules. These four molecules, at 5 μ M, increase the fluorescence quench rates statistically significantly in comparison to control ($p < 0.05$; $p < 0.01$ for MMV007384). (TIFF)

S3 Fig. MMV007384 is toxic in many assays of cell toxicity. MMV007384 is highly active in toxicity assays done by GNF-Novartis. The figure depicts the fluorescence quench rates as a

function of each compound's IFI Promiscuity Index (the Inhibition Frequency Index is the fraction of the HTS screens where a compound has been used and showed more than 50% inhibition). MMV007384 (marked in red) stands out by having the highest IFI Promiscuity Index (>0.25) and the largest increase in quench rate. IFI Promiscuity Indices for the different molecules were from Collaborative Drug Discovery, Inc. (<http://collaborativedrug.com> Burlingame, CA 94010).

(EPS)

S4 Fig. MMV007384 is highly toxic to HuH7 cells. Each molecule's cytotoxicity was quantified by its CC_{50} (the concentration, in μM , required to inhibit 50% of cell growth); MMV007384 is marked in red. Most of the Malaria Box compounds have CC_{50} 's on HuH7 cells (a human hepatocellular carcinoma line <http://huh7.com/>) less than 0.15. Only compounds that were tested by Novartis-GNF are plotted in this graph. CC_{50} values were from Collaborative Drug Discovery, Inc. (<http://collaborativedrug.com>, Burlingame, CA 94010).

(EPS)

S5 Fig. Malaria Box compounds' QED scores. The Malaria Box compounds tend to have "attractive" QED scores (mean = 0.619, median = 0.666), with no apparent difference between weighted and unweighted scores (not shown). Vertical dotted lines represent reported averaged of, from L to R—unattractive (complex), unattractive (simple), and attractive QED values. The red arrow marks MMV007384. Weighted QED did not differ substantially from unweighted QED (not shown). QED values were calculated using Pipeline Pilot (Accelrys, Inc., San Diego, CA) following [43].

(EPS)

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Author Contributions

Conceived and designed the experiments: NBR OSA. Performed the experiments: NBR. Analyzed the data: NBR OSA. Contributed reagents/materials/analysis tools: NBR OSA. Wrote the paper: NBR OSA.

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