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Parasitology | Short Form

A picomolar inhibitor of the *Plasmodium falciparum* IPP pathway

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ABSTRACT We identified MMV026468 as a picomolar inhibitor of blood-stage *Plasmodium falciparum*. Phenotyping assays, including isopentenyl diphosphate rescue of parasite growth inhibition, demonstrated that it targets MEP isoprenoid precursor biosynthesis. MMV026468-treated parasites showed an overall decrease in MEP pathway intermediates, which could result from inhibition of the first MEP enzyme DXS or steps prior to DXS such as regulation of the MEP pathway. Selection of MMV026468-resistant parasites lacking DXS mutations suggested that other targets are possible. The identification of MMV026468 could lead to a new class of antimalarial isoprenoid inhibitors.

KEYWORDS malaria, drug discovery, isoprenoid biosythesis, antimalarial agents, apicoplast, MEP pathway

Malaria is a devastating parasitic disease that remains a major global cause of morbidity and mortality (1). Antimalarials with new modes of action are urgently needed in the face of resistance to frontline drugs (2). Isoprenoid precursor biosynthesis is an attractive target for antimalarial drug development due to its essentiality and specificity in apicomplexan parasites (3). Unlike most eukaryotes which utilize the MVA pathway to synthesize isopentenyl diphosphate (IPP), *Plasmodium* employs the bacterial MEP/DOXP pathway. As such, all seven enzymes in the MEP pathway are absent in human cells, minimizing potential off-target toxicity by compounds targeting these enzymes (4). Consistent with this, fosmidomycin, an inhibitor of the MEP pathway enzyme DXR, tested in phase I and II human malaria trials, was well tolerated when administered either orally or subcutaneously and demonstrated a parasite clearance time <48 h (3, 5–7). Unfortunately, fosmidomycin had a short serum half-life and poor oral bioavailability (3, 6, 8), which may have contributed to recrudescent infection in 50% of patients (6).

New chemical scaffolds that target *Plasmodium* isoprenoid pathways could overcome the unfavorable drug properties of known inhibitors. Previously, we demonstrated that growth inhibition of blood-stage *P. falciparum* by compounds that inhibit the MEP pathway can be "rescued" by exogenous supplementation of IPP in the growth media (9, 10). We screened three open-access compound libraries released by the Medicines for Malaria Venture to identify new antimalarial compounds targeting IPP biosynthesis. The Pathogen Box and Pandemic Response Box each consisted of 400 structurally diverse compounds targeting a variety of neglected infectious diseases. The Covid box contained 160 compounds with known or predicted activity against coronaviruses. None of these compounds had a known mechanism of action (11). We tested the compounds for growth inhibition of *P. falciparum* W2 blood cultures at eight concentrations (0.05–6.7 µM) in the presence and absence of 200 µM IPP using a high-throughput SYBR-green assay (12) (Table S1). MMV026468 (Fig. 1A) demonstrated an IPP rescue of parasite growth inhibition at all eight concentrations. In a more accurate flow cytometry-based assay, the EC_{50} of MMV026468 was 850 pM (729–965 pM) for 72 h *P. falciparum* W2 growth inhibition which increased to 5.50 µM in the presence of IPP (Fig. 1B) (Table S2).

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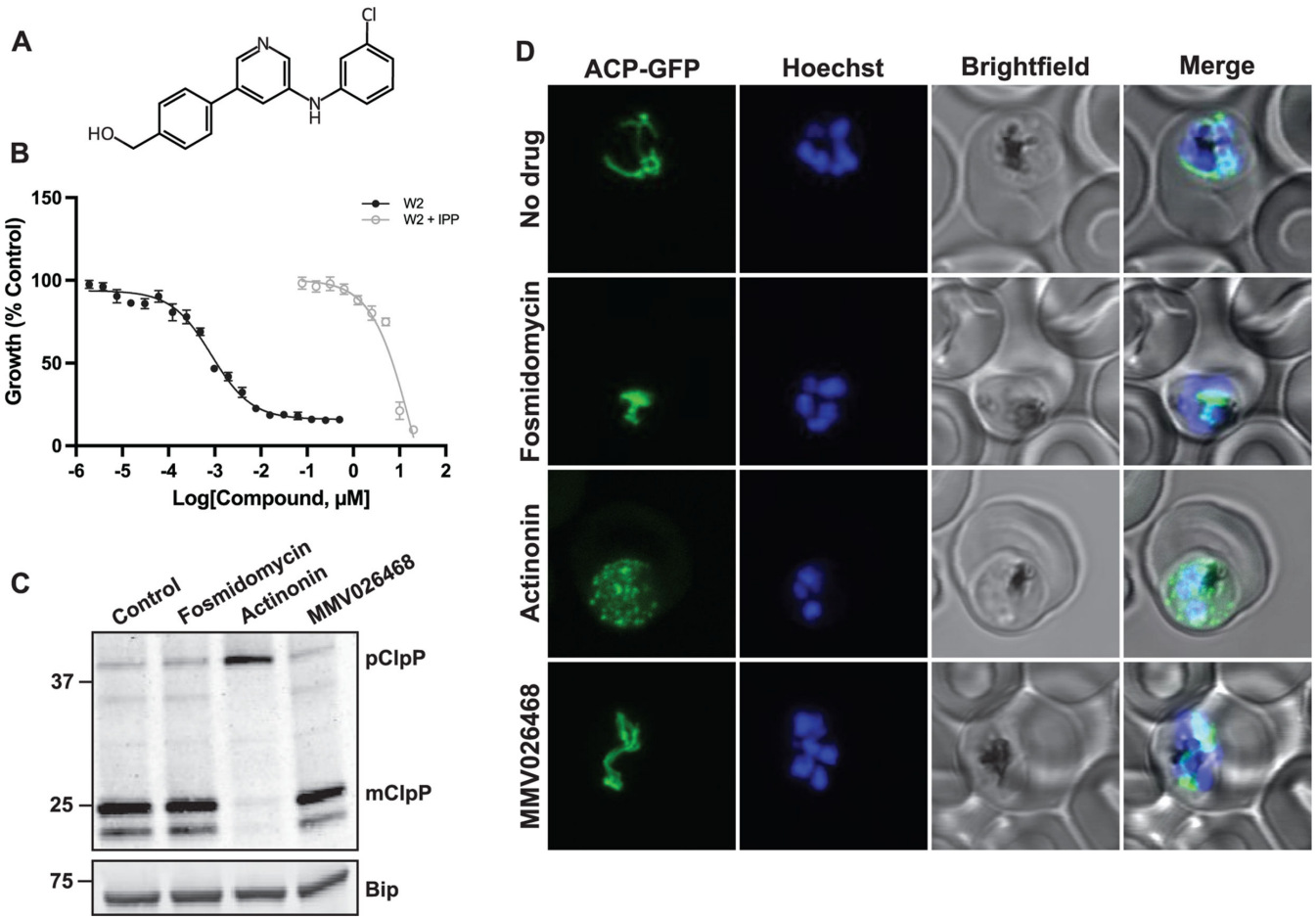

FIG 1 MMV026468 is a potent isoprenoid precursor pathway inhibitor. (A) Structure of MMV026468. (B) Seventy-two hour dose-dependent growth inhibition of blood-stage *P. falciparum* W2 in the presence and absence of IPP. Results are the mean and 95% CI of three independent experiments after subtraction of background fluorescence of uninfected RBCs. Growth is normalized relative to untreated controls. (C) Western blot of apicoplast targeted ClpP in the presence of actinonin/IPP, Fosmidomycin/IPP, MMV026468/IPP, and an untreated control. Full-length ClpP is ~40 kDA (pClpP), while mature ClpP (mClpP) after removal of its transit peptide is ~25 kDA. Bip serves as a loading control. (D) Representative live-cell fluorescent images of *Plasmodium* parasites expressing ACP_L-GFP in untreated, actinonin/IPP, Fosmidomycin/IPP, and MMV026468/IPP conditions.

The IPP rescue phenotype can be attributed to direct disruption of the IPP pathway or indirect disruption of the apicoplast, the plastid organelle where the IPP pathway is localized (9). To differentiate between these two mechanisms, we tested for the presence of an intact apicoplast which will exhibit a typical branched morphology in the schizont stage which can be visualized with an apicoplast-targeted GFP, Dd2 ACP_L-GFP. Intact apicoplast function can also be assessed by cleavage of targeting sequences from proteins, such as ClpP, imported into the organelle (Fig. 1C and D). Compounds that directly inhibit the IPP pathways, such as fosmidomycin, show normal morphology and protein processing (Fig. 1C and D). In contrast, compounds that disrupt the apicoplast resulting in organelle loss, such as actinonin, show abnormal ACP_L-GFP localization to punctate structures as previously described and the absence of ClpP processing (13, 14). Upon treatment with MMV026468, the apicoplast remained morphologically intact and retained its protein processing function suggesting that MMV026468 directly inhibits the IPP pathway (Fig. 1C and D).

To identify the step at which the pathway is inhibited, we performed targeted metabolomic profiling of MEP pathway intermediates in untreated, fosmidomycin-treated, and MMV026468-treated parasites (Fig. 2A). This method utilizes LC-MS/MS in combination with multiple reaction monitoring to quantify multiple pathway

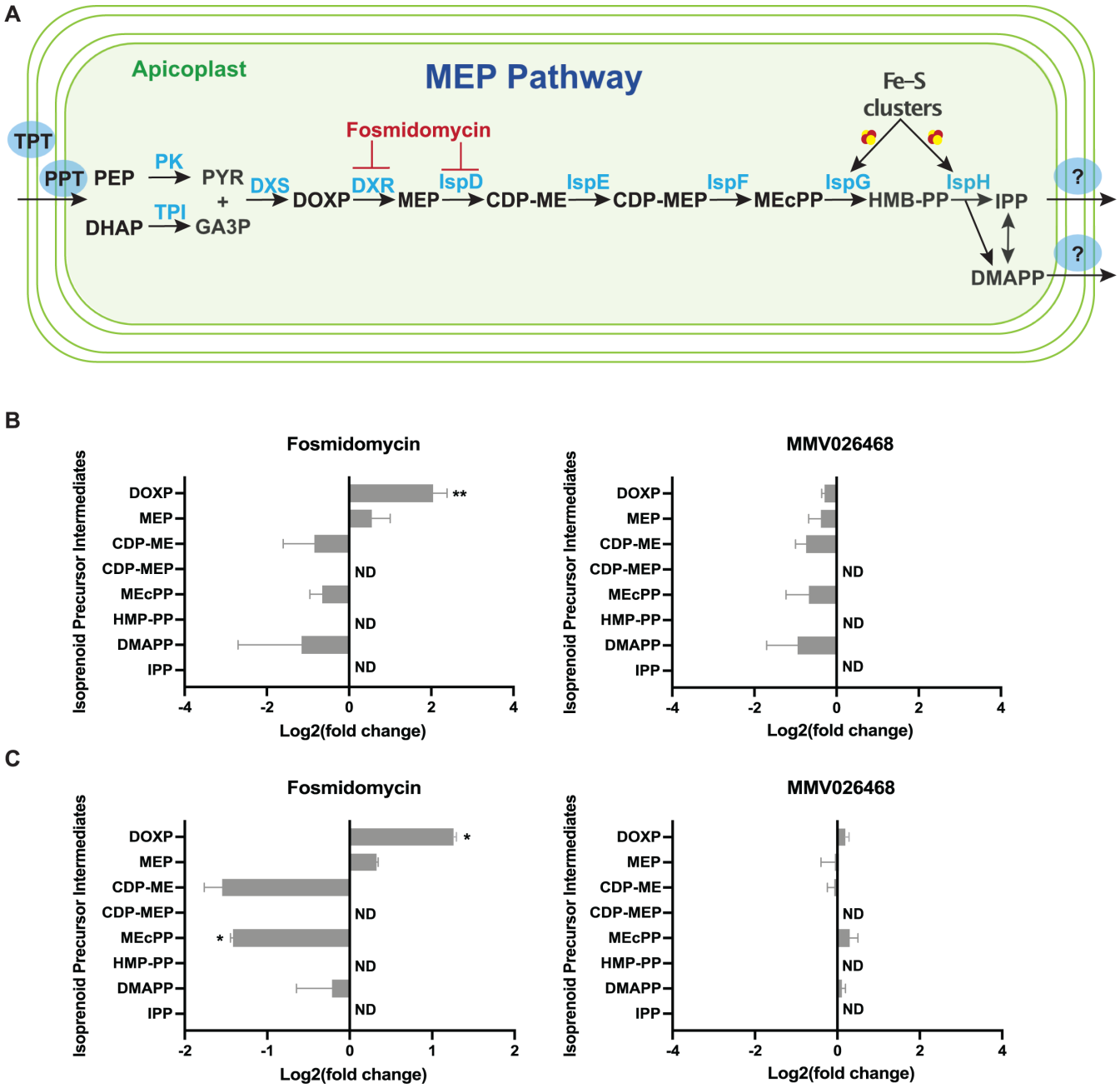

FIG 2 Targeted validation of isoprenoid precursor pathway genes and Fe-S pathway genes. (A) Overview of the non-mevalonate isoprenoid precursor biosynthesis pathway (DOXP/MEP) localized in the apicoplast. (B and C) Relative abundance of MEP pathway intermediates in W2 (B) or W2 MMV026468-resistant parasites (C) when treated with fosmidomycin or MMV026468 (compared to no treatment control) following a 30-h incubation initiated in early ring stage parasites. Data represent two independent experiments with error bars denoting ±SEM. *$P < 0.05$; **$P < 0.005$.

intermediates (15). In untreated parasites, DOXP, MEP, CDP-ME, MEcPP, and DMAPP, the products of DXS, DXR, IspD, IspF, and IspH respectively, were quantified, while CDP-MEP, HMB-PP, and IPP, corresponding to the activities of IspE, IspG, and IspH, were too low to be detected. For fosmidomycin and MMV026468 treated samples, 5 µM and 200 nM, respectively, were added to synchronized W2 parental or resistant early ring-stage cultures and incubated for 30 h. Lethal doses of drug were chosen to increase the likelihood of eliciting responses due to the direct effects of drug treatment related to the mechanism-of-action of the compound (16) rather than compensatory responses

described to occur with sublethal treatment as in reference (17). Since stalling of parasite morphologic development is observed starting at 40 h of drug treatment for both fosmidomycin and MMV026468 (Fig. S2A), we treated for 30 h to preferentially detect direct drug effects rather than general toxic effects due to parasite death.

Consistent with previous results, treatment with fosmidomycin, which inhibits DXR and partially targets IspD, resulted in an accumulation of their substrates, DOXP and MEP, and decrease in downstream MEP intermediates (Fig. 2B; Table S3) (15). In comparison, treatment with MMV026468 resulted in no clear build-up of any intermediates. Instead, we observed decreases in all detectable MEP intermediates (though the individual decreases did not reach $P < 0.05$ with two biological replicates) (Fig. 2B). This metabolite pattern could be consistent with direct inhibition of the first MEP enzyme DXS. Indirect inhibition of DXS could occur through negative feedback regulation by IPP and DMAPP, but is unlikely to be the mechanism of MMV026468 since DMAPP levels are decreased during treatment (18). The metabolite pattern could also be consistent with inhibiting a step prior to DXS, such as the production or import of its substrates pyruvate and glyceraldehyde 3-phosphate, but these glycolytic intermediates are necessary for energy production in the apicoplast and cytoplasm, as such, disruption of either inner and outer membrane transporters or apicoplast-localized pyruvate kinase has been shown to result in apicoplast loss (19). Finally MMV026468 could target regulation of the MEP pathway to cause a general decrease in pathway activity. HAD1 and HAD2 are cytoplasmic sugar phosphatases that dephosphorylate a number of glycolytic intermediates upstream of the MEP pathway, although studies have demonstrated that disruption of HAD1 or HAD2 results in an increase in MEP intermediates (20, 21).

To obtain genetic evidence for MMV026468's mechanism of action, we selected resistant parasites from clonal 3D7, W2, and Dd2 strains exposed to a lethal dose of MMV026468, 2 µM and 200 nM, respectively (Fig. 3A and B; Fig. S1A; Table S2). MMV026468-resistant W2 and Dd2 clones demonstrated nearly 10,000-fold greater EC_{50} values, whereas the MMV026468-resistant 3D7 population demonstrated a moderate 25-fold increase in EC_{50}. Resistance to MMV026468 is maintained without drug for >6 months in culture and following cryopreservation (Fig. 3C and D; Fig. S1B). The stability of the resistance phenotype suggested that the change leading to decreased drug susceptibility is genetically encoded (22). Strains resistant to MMV026468 no longer demonstrated a decrease in MEP intermediates upon MMV026468 treatment but continue to show the expected pattern upon treatment with fosmidomycin (Fig. 2C). MMV026468-resistant parasites did not show altered susceptibility to four other antimalarials, including fosmidomycin or artemisinin, such that the resistance mechanism is specific to MMV026468 (Fig. 3E through H, Fig. S1C through M; Table S2). Of note, W2 and Dd2 are inherently resistant to pyrimethamine and chloroquine. Two W2-resistant clones, two Dd2 resistance clones, 3D7-resistant population, and all three parental stains were subjected to whole genome sequencing. Compared to their parental clones, approximately 10–200 single nucleotide variants (SNVs) and indels were detected in MMV026468-resistant parasites but no copy number variants (Table S4). No genetic changes were detected in any known isoprenoid biosynthesis genes, including DXS or HAD1/HAD2. None of the genetic changes were shared across all resistant strains. Unlike other published compounds which exhibited a few, distinct SNVs in drug-resistant strains, we were unable to identify a genetic change specifically associated with MMV026468 resistance. Transcriptomic profiling of resistant parasites also did not reveal specific changes. All sequencing data are available on the NCBI Sequence Read Archive as BioProject accession PRJNA1091333.

Overall, MMV026468 is a potent picomolar inhibitor of *P. falciparum* IPP biosynthesis, a validated, high-priority antimalarial mechanism of action. Metabolite profiling of the MEP pathway suggests it may inhibit the first enzyme DXS, though alternative targets that could not be identified in resistant parasites may also be possible. In supporting documentation that accompanied the Pathogen Box, MMV026468 had low mammalian cell cytotoxicity with HepG2 (human hepatocellular carcinoma) CC_{20} of 20.5 µM and

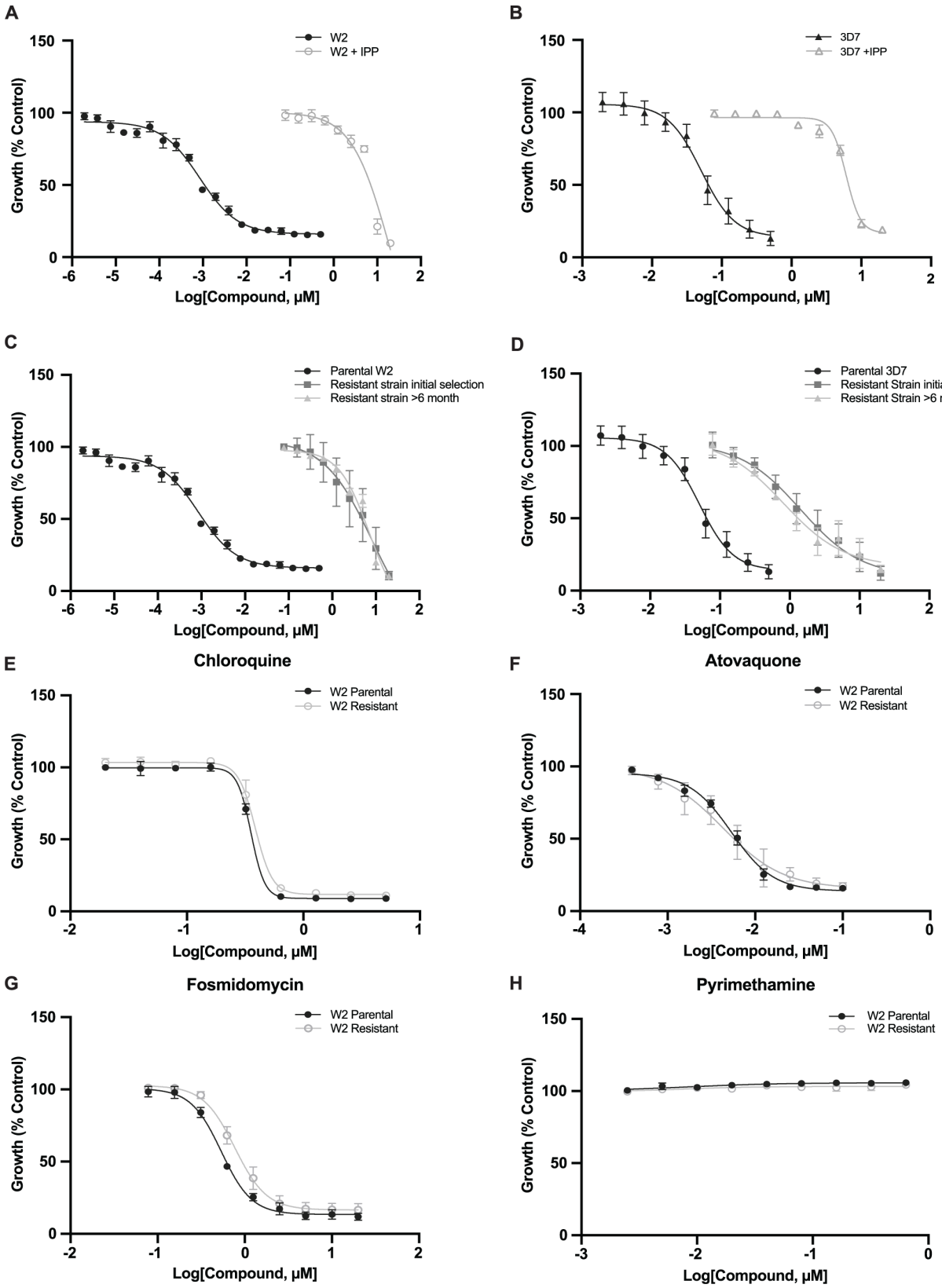

FIG 3 *In vitro* selected resistant parasites show stable, specific, an <10,000× fold change in resistance. (A and B) Seventy-two hour dose-dependent growth inhibition of parental strains in the presence and absence of IPP. Results are the mean and 95% CI of three independent experiments after subtraction of background fluorescence of uninfected RBCs. Growth is normalized relative to untreated controls. (C and D) Seventy-two hour dose-dependent growth inhibition

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FIG 3 (Continued)

of parental strains, resistant strains after initial selection, and resistant strains cultured for 6 months after removal of MMV026468 drug pressure. Results are the mean and 95% CI of three independent experiments after subtraction of background fluorescence of uninfected RBCs. Growth is normalized relative to untreated controls. (E–H) Seventy-two hour dose-dependent growth inhibition of parental and resistant W2 parasites against non-isoprenoid inhibitors: chloroquine, atovaquone, and pyrimethamine, as well as isoprenoid inhibitor, fosmidomycin. Results are the mean and 95% CI of three independent experiments after subtraction of background fluorescence of uninfected RBCs. Growth is normalized relative to untreated controls.

HL60 (human leukemia) CC_{50} of 25 µM. Moreover, artemisinin-resistant K13 mutant strains remained susceptible to MMV026468 ensuring efficacy in the setting of clinical artemisinin resistance (Fig. S1N). The identification of MMV026468 provides a strong lead candidate, which with additional medicinal chemistry optimization, could lead to a new class of antimalarial isoprenoid inhibitors to counter the spread of drug-resistant parasites.

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ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental material (AAC01238-23-s0001.pdf). Supplemental methods; Fig. S1 and S2.
Table S1 (AAC01238-23-s0002.xlsx). Results of *P. falciparum* growth inhibition and IPP rescue for Pathogen, Pandemic Response, and COVID Box compound libraries.
Table S2 (AAC01238-23-s0003.tiff). EC_{50} values for all dose-response assays.
Table S3 (AAC01238-23-s0004.tiff). Metabolomics data.
Table S4 (AAC01238-23-s0005.xlsx). SNV analysis for resistant parasites.

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