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In Vitro Antimalarial Activity of Different Inhibitors of the Plasmodial Isoprenoid Synthesis Pathway

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Previous studies have shown that fosmidomycin, risedronate, and nerolidol exert antimalarial activity *in vitro*. We included squalestatin, an inhibitor of the isoprenoid metabolism in *Erwinia uredovora*, and found that combinations of compounds which act on different targets of the plasmodial isoprenoid pathway possess important supra-additivity effects.

Malaria expansion in some areas has been attributed to the failure of vector-control policies and, mainly, to the increase of parasite resistance to drugs commonly used for its therapy (1). This alarming scenario has fuelled research in new antimalarial drugs, and, with the sequencing of parasite genomes, new potential drug targets have emerged. Burrows et al. published a review discussing the strategies for designing the next generation of medicines for malaria control (2). A metabolic pathway that may be a likely target for evaluating potential antimalarials is the isoprenoid pathway. The first intermediates are biosynthesized in the apicoplast, which is an organelle present in protozoan parasites of the subphylum Apicomplexa, including Plasmodia (3).

We have previously demonstrated that the isoprenoid biosynthesis pathway is functionally active in the intraerythrocytic stages of *Plasmodium falciparum* and that final products of this pathway such as dolichol of 11 to 12 isoprenic units (4), ubiquinones (5), isoprenylated/dolichylated proteins, carotenoids, menaquinone, and tocopherol are biosynthesized by this parasite (6). Several inhibitors of enzymes of the methyl erythritol diphosphate (MEP) and isoprenoid pathways were described as potential antimalarial drugs (6).

Our aim in this work was to identify and validate different targets in the isoprenoid pathway in *P. falciparum* intraerythrocytic stages. We evaluated the combination of four drugs, three of them (fosmidomycin, nerolidol and risedronate) inhibiting parasite growth at some point in the MEP/isoprenoid pathway in intraerythrocytic stages of *P. falciparum* (6, 7, 8). We also evaluated the effect of squalestatin on *P. falciparum* growth. This drug is an inhibitor of the phytoene synthase in *Erwinia uredovora* (9, 10). Importantly, an enzyme with phytoene synthase activity was described in intraerythrocytic stages of *P. falciparum* (11).

Parasites of *P. falciparum* clone 3D7 were cultured according to the protocol described by Trager and Jensen (12) where human serum was replaced by Albumax I (0.5%) (4). Stocks (1 mM) of squalestatin, risedronate, and fosmidomycin were prepared in RPMI medium, whereas nerolidol was diluted in ethanol, and all drugs were stored at −70°C.

Assays were performed in 96-well microtiter plates containing 100 μl of 3D7 culture at 1% parasitemia and 3% hematocrit and 100 μl of the appropriate drug or drug combination in RPMI medium. Parasite growth was determined using Giemsa-stained smears immediately before the start of the assay and at intervals of 24 to 48 h. Vehicle controls were included on each plate. All tests were performed in triplicate for three independent experiments.

Values for 50% inhibitory concentrations (IC_{50}s) for the compounds studied alone or in pairs were obtained from concentration-response curves, by means of fitting a sigmoid function to the original points using OriginPro version 8.5 software (8). Concentration-response curves were constructed using seven dilutions of solutions containing either an isolated drug or a combination of 2 drugs. Solutions of the combinations were prepared by mixing one IC_{50} of one drug with one IC_{50} of another.

Fractional analysis was performed using the following equation, considering two hypothetical drugs, A and B:

$$C_A/IC_{50}(A) + C_B/IC_{50}(B)$$

where C_A is the concentration of drug A in the IC_{50} of the mixture of drugs A and B, $IC_{50}(A)$ is the 50% inhibitory concentration of drug A, and $C_A/IC_{50}(A)$ is the fraction of the $IC_{50}(A)$ that drug A contributes to the IC_{50} of the mixture of drugs A and B. The same definitions are applicable to drug B. The sum of the fractions of $IC_{50}(A)$ and $IC_{50}(B)$ indicates whether a mixture induces additive, supra-additive, or subadditive effects, instances which correspond to the respective result of the sum of the fractions of IC_{50} of 1, <1, or >1. Confidence intervals (95% CI) were constructed for the sum of the fractions of IC_{50} in order to evaluate, statistically, whether a sum differed significantly from 1 (13).

Isobolographic analysis was also used to evaluate whether the effects of the drugs in each mixture combined in an additive, supra-additive, or subadditive fashion (13, 14, 15). Confidence intervals (95% CI) were calculated for the averages of the IC_{50}(s) of the drugs employed alone. These intervals were used to draw lines above and below the line corresponding to additivity of the isobologram, thus defining confidence limits for this line. The points in the graph corresponding to the average IC_{50}s of the mixtures were also plotted together with 95% CI (Fig. 1) (13). This permitted us to employ the following criteria: points in the isobolograph with

Received 25 August 2014 **Returned for modification** 3 December 2014
Accepted 4 June 2015

Accepted manuscript posted online 8 June 2015

Citation da Silva MF, Saito AY, Peres VJ, Oliveira AC, Katzin AM. 2015. *In vitro* antimalarial activity of different inhibitors of the plasmodial isoprenoid synthesis pathway. Antimicrob Agents Chemother 59:5084–5087. doi:10.1128/AAC.04161-14.

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doi:10.1128/AAC.04161-14

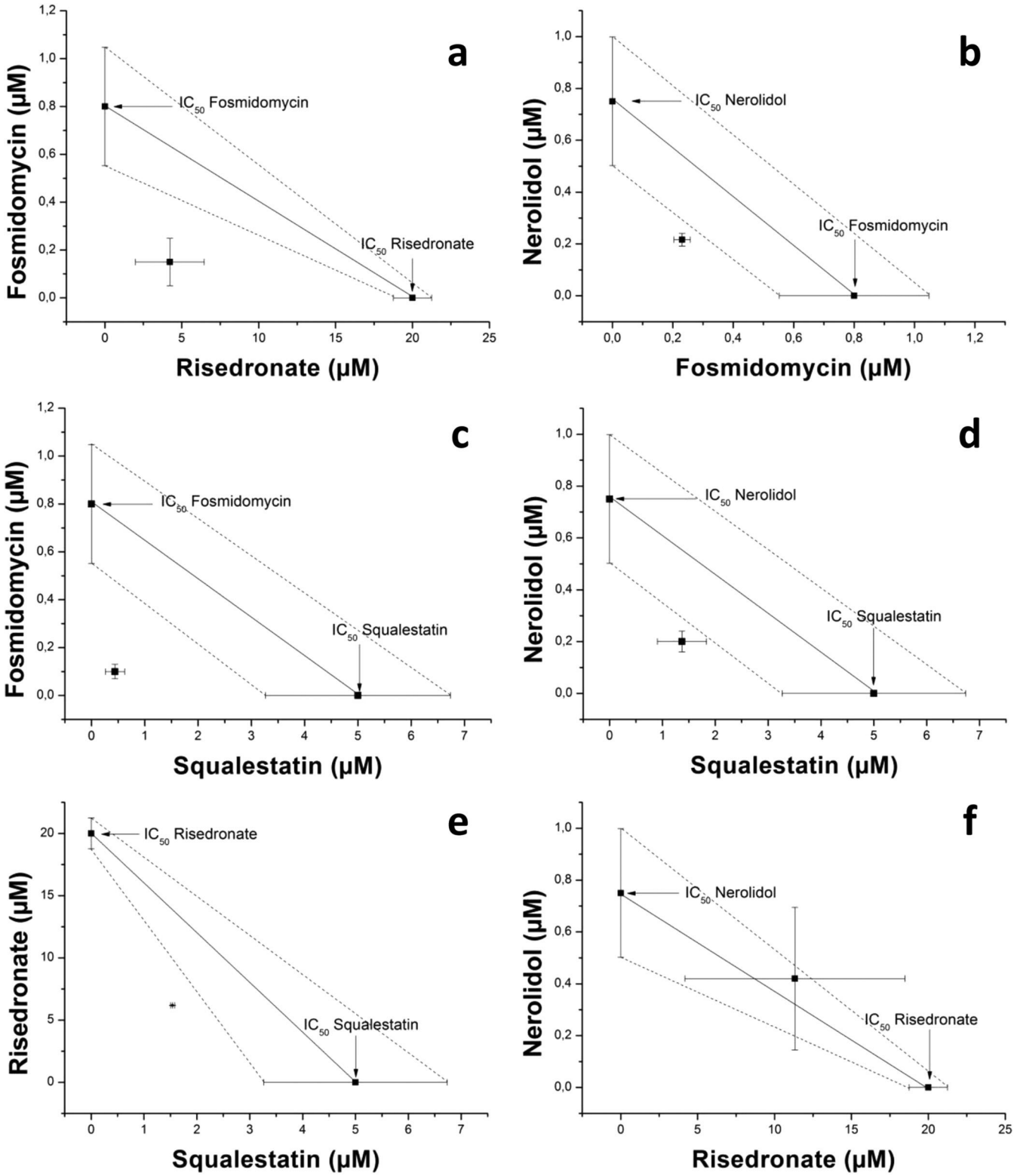

FIG 1 Isobolograms for the interaction of the studied compounds. In all panels, the solid line connecting the single-drug average IC_{50} is the theoretical additive line and the dashed lines below and above this line correspond to the 95% CI. The average IC_{50}s of the mixtures were also plotted together with the 95% CI.

TABLE 1 Values of the IC_{50} of the compounds, applied alone or in association, sum of fractions of the IC_{50}, and type of interaction of the compounds applied in association

Compound(s)	IC_{50}(s) (arithmetic mean ± SD) (μM) (no. of expts replicated in each group)	Sum of IC_{50} fractions (95% CI[a])	Type of interaction
Squalestatin	5.0 ± 0.6 (3)		
Fosmidomycin	0.8 ± 0.1 (3)		
Nerolidol	0.75 ± 0.10 (3)		
Risedronate	20.0 ± 0.5 (3)		
Fosmidomycin/risedronate	0.16 ± 0.04/4.23 ± 0.90 (3)	0.40 (0.65–0.17)	Supra-additive
Nerolidol/fosmidomycin	0.22 ± 0.01/0.23 ± 0.01 (3)	0.57 (0.65–0.51)	Supra-additive
Fosmidomycin/squalestatin	0.10 ± 0.03/0.44 ± 0.02 (3)	0.22 (0.31–0.11)	Supra-additive
Nerolidol/squalestatin	0.21 ± 0.04/1.41 ± 0.31 (3)	0.54 (0.65–0.51)	Supra-additive
Risedronate/squalestatin	6.19 ± 0.07/1.54 ± 0.02 (3)	0.62 (0.64–0.60)	Supra-additive
Nerolidol/risedronate	0.42 ± 0.11/11.32 ± 2.88 (3)	1.13 (1.84–0.40)	Subadditive

[a] CI, confidence interval.

confidence intervals not overlapping the confidence interval of the line of additivity indicated a supra-additive combination, a potentiating combination (if the point is located below the line of additivity), or a subadditive antagonistic combination (if the point is located above the line of additivity).

Table 1 displays the IC_{50}s (μM) of the compounds, employed alone or in association. The sums of the fractions of IC_{50}, in the cases of the compounds used in association, are also displayed. Table 1 is especially informative with regard to the sums of IC_{50} fractions, which constitute the basis of the fractional analysis. Thus, as shown in Table 1, in the cases of the associations of fosmidomycin and risedronate, nerolidol and fosmidomycin, fosmidomycin and squalestatin, nerolidol and squalestatin, and risedronate and squalestatin, the confidence intervals did not contain 1, indicating that these sums of the fractions of IC_{50} are significantly smaller than 1. Therefore, these associations exhibited supra-additive interactions of compounds in the mixture. The association of nerolidol and risedronate suggests a subadditive interaction because the corresponding confidence interval for the sum of the fractions of IC_{50} contains 1.

Regarding the isobolographic results, it was observed (Fig. 1) that, in 5 of the 6 combinations in the present work (fosmidomycin and nerolidol, fosmidomycin and risedronate, fosmidomycin and squalestatin, squalestatin and nerolidol, and squalestatin and risedronate), the confidence intervals of the combination did not overlap the confidence interval of the line of additivity. Furthermore, in all of these 5 cases, the points corresponding to the combinations fell below the line of additivity (Fig. 1). This means that the drugs produced effects that could be considered supra-additive in all of these combinations. The combination of nerolidol and risedronate displayed an isobolograph that could be indicative of a simple subadditive effect (Fig. 1).

Of the four drugs evaluated in this work, only fosmidomycin was tested in clinical trials in malaria patients. Synergistic activity *in vitro* between fosmidomycin and clindamycin against various strains of *P. falciparum* has been demonstrated previously (16). The results shown here can help us understand the interactions between the different intermediates of the MEP/isoprenoid pathway in *P. falciparum* intraerythrocytic stages.

The four drugs tested in this work act in different cellular compartments. Fosmidomycin inhibits 1-deoxy-D-xylulose 5-phosphate reductoisomerase (PfDXR), an enzyme localized in the apicoplast (7). Risedronate inhibits the enzyme farnesyl-pyrophosphate/geranylgeranyl-PP synthase, probably located in the cytoplasm (17). Nerolidol inhibits the enzyme octaprenyl phosphate/phytoene synthase, which is localized in the cytoplasm and also in mitochondria (11, 18). Squalestatin inhibits squalene synthase in mammalian cells, but this enzyme is probably absent in *Plasmodium* because malaria parasites apparently do not synthesize sterols (19). Nevertheless, squalestatin inhibits phytoene synthase in *Erwinia uredovora* (9), and the same effect could be occurring in *P. falciparum*.

Fosmidomycin inhibits PfDXR, which is the second enzyme in the MEP pathway. Yeh and DeRisi demonstrated that fosmidomycin inhibition can be chemically rescued by supplementation with isopentenyl pyrophosphate (IPP), the pathway product. Surprisingly, IPP supplementation also completely prevents death of parasites following treatment with antibiotics that cause loss of the apicoplast (20).

Another enzyme in the isoprenoid pathway that is probably important for parasite growth is the farnesyl-PP/geranylgeranyl-PP synthase. This assumption arises from experiments of "growth rescue" performed after treatment with risedronate where only farnesyl-PP and geranylgeranyl-PP restored the growth. Based on growth rescue and enzyme inhibition experiments, plasmodial farnesyl-PP/geranylgeranyl-PP synthase was shown to be a major target for risedronate (8).

Nerolidol inhibits several final products of the isoprenoid pathway in the intraerythrocytic stages of *P. falciparum*, namely, dolichols and ubiquinones, and also abrogates isoprenylation of proteins (21). The K_i of octaprenyl-PP synthase (the enzyme responsible for biosynthesis of the isoprenic chain of ubiquinone) for nerolidol is 10 nM (18). All three enzymes are related to isoprenoid biosynthesis.

The finding that these drugs displayed supra-additivity effects (Fig. 1a, b, c, d, and e) when combined might be due to the fact that they target different enzymes in the same biosynthetic pathway in *Plasmodium*.

Additionally, our group has shown that nerolidol inhibited the synthesis of several final products of the isoprenoid biosynthesis, such as coenzyme Q and dolichol, and also protein isoprenylation (6, 21). As risedronate is also an inhibitor of protein isoprenylation (8), the subadditive effect detected when risedronate is combined with nerolidol may indicate that the two compounds act at the same target of isoprenoid pathways.

The *in vitro* interactions between the drugs evaluated in this work provide an essential background for clinical studies. How-

ever, they do not necessarily determine the efficacy of a combination in the host, since this also depends on pharmacokinetic characteristics as mentioned by Fivelman et al. (22). Nonetheless, data obtained in this study provide additional evidence for the definition of the biochemical targets of these drugs in *Plasmodium* and might prove useful for drug development projects targeting the isoprenoid pathway in malaria.

ACKNOWLEDGMENTS

This work was supported by grants from CNPq and FAPESP (Brazil). M.F.D.S. is the recipient of a postgraduate fellowship from CAPES. A.Y.S. was the recipient of a postgraduate fellowship from FAPESP.

We thank S. Wendel (Blood Bank at the Sírio Libanês Hospital) for providing the erythrocytes and Emilia Kimura, Gerhard Wunderlich, and Silvia R. B. Uliana for critical reading of the manuscript.

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