AAC Journals.ASM.org

A Pantetheinase-Resistant Pantothenamide with Potent, On-Target, and Selective Antiplasmodial Activity

Cristiano J. Macuamule,[a] Erick T. Tjhin,[b] Collins E. Jana,[a] Leanne Barnard,[a] Lizbé Koekemoer,[a] Marianne de Villiers,[a] Kevin J. Saliba,[b,c] Erick Strauss[a]

Department of Biochemistry, Stellenbosch University, Stellenbosch, South Africa[a]; Research School of Biology, College of Medicine, Biology, and Environment, The Australian National University, Canberra, ACT, Australia[b]; Medical School, College of Medicine, Biology, and Environment, The Australian National University, Canberra, ACT, Australia[c]

Pantothenamides inhibit blood-stage *Plasmodium falciparum* with potencies (50% inhibitory concentration [IC_{50}], ~20 nM) similar to that of chloroquine. They target processes dependent on pantothenate, a precursor of the essential metabolic cofactor coenzyme A. However, their antiplasmodial activity is reduced due to degradation by serum pantetheinase. Minor modification of the pantothenamide structure led to the identification of α-methyl-*N*-phenethyl-pantothenamide, a pantothenamide resistant to degradation, with excellent antiplasmodial activity (IC_{50}, 52 ± 6 nM), target specificity, and low toxicity.

One-half of the world's population (~3.4 billion people) is at risk of contracting malaria, with pregnant women and children <5 years of age being especially vulnerable. In 2013, the WHO estimated that malaria caused ~584,000 deaths globally, with the majority occurring in Africa (1). Although efforts to control and to eliminate malaria in the past 15 years have saved an estimated 3.3 million lives (1), drug-resistant parasites continue to emerge (2). This places the progress in the fight against the disease under pressure, especially since there is no effective vaccine against malaria (3). Several new drug targets have been identified in recent years (4); however, these targets now need to be exploited through the development of directed treatments.

We are interested in targeting the biosynthesis of the essential cofactor coenzyme A (CoA) from the water-soluble vitamin B_5 (pantothenate, compound 1 in Fig. 1) for antimalarial drug development (5, 6). It has been shown that extracellular pantothenate is essential for intracellular malaria parasites (7), which indicates that *Plasmodium falciparum* does not utilize exogenous CoA but must synthesize CoA *de novo* (8).

Pantothenate analogues interfere with the ability of *P. falciparum* to utilize the vitamin, with many analogues being characterized as growth inhibitors of the blood-stage parasites (9–11). Furthermore, a recent study showed that CoA biosynthesis can be targeted by a chemically diverse set of inhibitors that do not resemble pantothenate, the most potent of which had a 50% inhibitory concentration (IC_{50}; the concentration that inhibits parasite proliferation by 50%) of 120 nM against blood-stage parasites (12). These studies support pantothenate utilization (and therefore CoA biosynthesis and CoA-dependent processes) as an antiplasmodial target.

Recently we showed that *N*-substituted pantothenamides (PanAms), a specific class of pantothenate analogues, have excellent antiplasmodial activity. Among these, *N*-phenethyl-pantothenamide (*N*-PE-PanAm) (compound 2 in Fig. 1) exhibited an IC_{50} of 20 nM (13); this potency is comparable to that of chloroquine (14, 15). In practice, however, the antiplasmodial activity of the PanAms is decreased since they are degraded by pantetheinase (13), a ubiquitous enzyme of the Vanin protein family that is present in serum (16, 17). Pantetheinase normally catalyzes the hydrolysis of pantetheine (a CoA-derived metabolite) to form pantothenate and cysteamine (18, 19), but it also acts on compounds with a wide range of variations in the cysteamine moiety, including the PanAms (Fig. 1) (13). In a previous study, we found that replacement of the β-alanine moiety of the PanAms with either glycine or γ-aminobutyric acid gave rise to pantetheinase-resistant variants, due to displacement of the scissile amide bond (20). Unfortunately, these structural modifications also reduced the potency of the resulting PanAms (IC_{50} values of ≥1 μM), indicating that their target (or targets) requires the pantothenate core structure to be retained for optimal inhibition.

In light of this finding, we set out to develop a pantetheinase-resistant PanAm in which the β-alanine core was retained. This was achieved by adding a methyl group to the carbon adjacent to the amide carbonyl group, thereby increasing the steric bulk at this center. We predicted that this modification would reduce the rate of pantetheinase-mediated hydrolysis by limiting the access of the enzyme's cysteine nucleophile to the scissile amide bond. The methylated version of *N*-PE-PanAm, i.e., α-methyl-*N*-PE-PanAm (α-Me-*N*-PE-PanAm) (compound 3 in Fig. 1), was prepared by condensing D,L-3-amino-isobutyrate to pantolactone, followed by partial purification by cation-exchange chromatography. The product, α-methyl-D-pantothenate, was purified by flash column chromatography (FCC) before being coupled to *N*-phenethylamine using diphenylphosphoryl azide in the presence of triethylamine. After purification by FCC, α-Me-*N*-PE-PanAm was

Received 9 December 2014 **Returned for modification** 4 January 2015
Accepted 2 April 2015

Accepted manuscript posted online 6 April 2015

Citation Macuamule CJ, Tjhin ET, Jana CE, Barnard L, Koekemoer L, de Villiers M, Saliba KJ, Strauss E. 2015. A pantetheinase-resistant pantothenamide with potent, on-target, and selective antiplasmodial activity. Antimicrob Agents Chemother 59:3666–3668. doi:10.1128/AAC.04970-14.

Address correspondence to Erick Strauss, estrauss@sun.ac.za.

C.J.M. and E.T.T. contributed equally to this article.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/AAC.04970-14.

doi:10.1128/AAC.04970-14

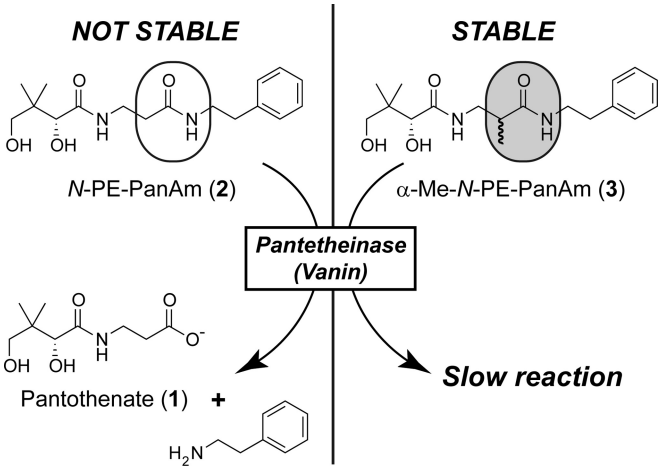

FIG 1 (Left) Structure of *N*-PE-PanAm (compound 2), which can be degraded by pantetheinase (Vanin) to form pantothenate (compound 1) and phenethylamine. (Right) Structure of α-Me-*N*-PE-PanAm (compound 3), which shows limited degradation by pantetheinase. The vulnerable (scissile) amide bond is indicated by the oval on the left, while the shaded oval on the right shows how it is modified by introduction of the methyl group to increase stability.

obtained in a final overall yield of 45%, as a mixture of two epimers (see the supplemental material for more details).

The antiplasmodial activity of α-Me-*N*-PE-PanAm against the chloroquine-sensitive *P. falciparum* strain 3D7 (chloroquine IC_{50}, 11 ± 1 nM [mean ± standard error of the mean {SEM}]; $n = 3$) was determined in "aged medium" (i.e., medium in which pantetheinase had been inactivated by incubation at 37°C for 40 h), in a manner similar to that used previously for *N*-PE-PanAm (13, 20). Under these conditions, α-Me-*N*-PE-PanAm showed excellent antiplasmodial activity, with an IC_{50} of 29 ± 2 nM (mean ± SEM; $n = 3$), a value that is only slightly greater than that of *N*-PE-PanAm (Fig. 2a). Furthermore, α-Me-*N*-PE-PanAm demonstrated exceptional resistance to degradation by pantetheinase, compared to *N*-PE-PanAm, as can be seen from its antiplasmodial activity in normal medium (i.e., with active pantetheinase), with an IC_{50} of 52 ± 6 nM (mean ± SEM; $n = 3$) (Fig. 2a), compared to the *N*-PE-PanAm IC_{50} of ~6,200 nM (13, 20). Performing the same test with a chloroquine-resistant strain (strain Dd2; chloroquine IC_{50}, 173 ± 5 nM [mean ± range/2]; $n = 2$) gave an IC_{50} of 129 ± 4 nM (mean ± range/2; $n = 2$); based on currently available data, it is unclear whether this difference is related to chloroquine resistance or is merely a variation in strain sensitivity. More importantly, resistance to pantetheinase degradation did not come at a cost in target specificity, since addition of excess extracellular pantothenate (100 μM) to the medium antagonized the antiplasmodial activity of α-Me-*N*-PE-PanAm against the 3D7 strain (IC_{50}, 860 ± 102 nM [mean ± SEM]; $n = 3$; $P = 0.01$) (Fig. 2a).

To confirm the stability of α-Me-*N*-PE-PanAm, we also tested its *in vitro* degradation by recombinant pantetheinase (human VNN1) (Fig. 2b). This was done by incubating substrate (500 μM *N*-PE-PanAm or α-Me-*N*-PE-PanAm; 500 μM phenethylamine was used as a reference, i.e., equivalent to 100% product formation) in 100 mM HEPES (pH 7.6) containing 500 μM dithiothreitol (DTT) and 0.05 μg/μl bovine serum albumin (BSA), at 37°C. The reaction (in a final volume of 300 μl) was initiated by the addition of pantetheinase (1.6 μg/μl), and the mixture was incubated for 24 h. The amount of amine produced was determined by quenching 30 μl of the reaction mixture with 10 μl of *N*-ethylmaleimide (6 μM), followed by incubation (for 10 min at 37°C) with 2 mM fluorescamine in 517 mM borate (pH 9), in a final volume of 145 μl. Fluorescence was subsequently measured using a Thermo Varioskan multiplate spectrofluorimeter (excitation wavelength, 395 nm; emission wavelength, 485 nm). We were able to confirm that α-Me-*N*-PE-PanAm was more resistant to pantetheinase-mediated degradation than *N*-PE-PanAm, as it showed only 26% ± 2% (mean ± range/2; $n = 2$) hydrolysis (normalized to the control, which represented 100% phenethylamine formed) after 24 h, compared to 96% ± 9% (mean ± range/2; $n = 2$) for *N*-PE-PanAm under the same conditions.

The activity of α-Me-*N*-PE-PanAm was tested against a human cell line (human foreskin fibroblasts [HFF]) to determine its selectivity (21). The cells were exposed to α-Me-*N*-PE-PanAm

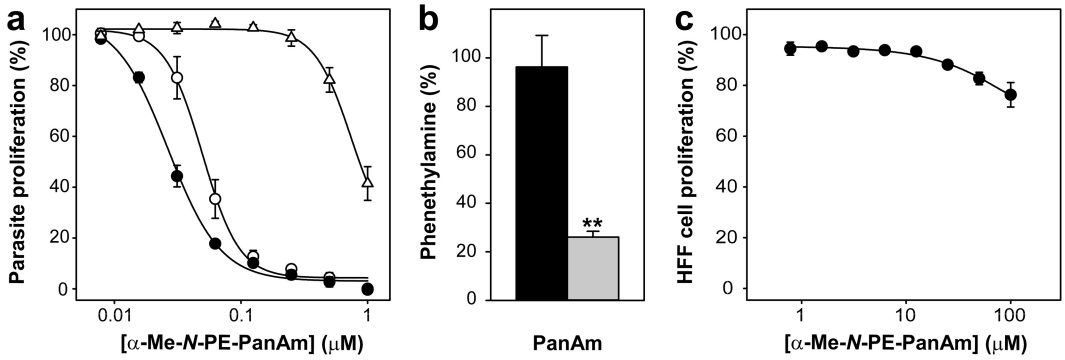

FIG 2 (a) Inhibition of proliferation of *P. falciparum* parasites (chloroquine-sensitive strain 3D7) by α-Me-*N*-PE-PanAm. Parasites were cultured for 96 h in medium with (○) or without (●) pantetheinase activity; the inhibition was antagonized when the extracellular pantothenate concentration in the medium with pantetheinase was increased from the usual 1 μM to 100 μM (△), consistent with the compound being on target. Values represent the mean ± SEM of three independent experiments, each performed in triplicate. (b) Pantetheinase-mediated hydrolysis of *N*-PE-PanAm (black bar) and α-Me-*N*-PE-PanAm (gray bar) *in vitro* after treatment with recombinant human pantetheinase for 24 h. The amount of phenethylamine released was determined by derivatization with fluorescamine. Values represent the mean from two independent experiments, each performed in triplicate; the error bars represent range/2. **, $P < 0.001$, Student's *t* test. (c) HFF proliferation in the presence of α-Me-*N*-PE-PanAm after 96 h. Values represent the mean ± SEM of three independent experiments, each performed in triplicate.

(0.781 to 100 μM) for 4 days to reach confluence, and plates were stored at −80°C prior to exposure to SYBR Safe, as was done for the parasite experiments. We found that α-Me-*N*-PE-PanAm had limited cytotoxicity for HFF cells, with a selectivity index (SI) greater than 1,500 (Fig. 2c), rivalling the SI of chloroquine (~1,300) determined using a similar cell line (15).

With these promising findings, we unveil α-Me-*N*-PE-PanAm as the first pantothenate analogue with excellent potential as a new lead compound for antimalarial drug development, based on its potent inhibition of blood-stage parasites in the presence of serum pantetheinase, lack of activity against human cells, and desirable physicochemical characteristics (22) (see the supplemental material for details). Future work will focus on determining whether the two epimers show a difference in activity, performing tests on other stages of the parasite's life cycle, and determining the pharmacokinetic properties and *in vivo* efficacy of α-Me-*N*-PE-PanAm, to establish its long-term potential for development as an antimalarial.

ACKNOWLEDGMENTS

Aspects of this work were supported by a grant from the South African Malaria Initiative to K.J.S. and E.S. Australia Awards Small Grants Scheme, an initiative of the Australian Government (to C.J.M.), made visits to the Australian National University possible and are gratefully acknowledged. C.J.M. was supported by a Canon Collins Trust Scholarship. E.T.T. was supported by an International Postgraduate Research Scholarship from the Australian government. C.E.J., L.B., and L.K. were supported by bursaries and fellowships from the National Research Foundation of South Africa. M.D.V. was supported by the National Research Foundation of South Africa as a Research Career Advancement Fellow.

We are grateful to the Canberra Branch of the Australian Red Cross Blood Service for the provision of red blood cells and the van Dooren laboratory at the Australian National University for providing the HFF cells.

REFERENCES

1. **World Health Organization.** 2014. World malaria report 2014. World Health Organization, Geneva, Switzerland.
2. **Miotto O, Almagro-Garcia J, Manske M, Macinnis B, Campino S, Rockett KA, Amaratunga C, Lim P, Suon S, Sreng S, Anderson JM, Duong S, Nguon C, Chuor CM, Saunders D, Se Y, Lon C, Fukuda MM, Amenga-Etego L, Hodgson AV, Asoala V, Imwong M, Takala-Harrison S, Nosten F, Su XZ, Ringwald P, Ariey F, Dolecek C, Hien TT, Boni MF, Thai CQ, Amambua-Ngwa A, Conway DJ, Djimde AA, Doumbo OK, Zongo I, Ouedraogo JB, Alcock D, Drury E, Auburn S, Koch O, Sanders M, Hubbart C, Maslen G, Ruano-Rubio V, Jyothi D, Miles A, O'Brien J, Gamble C, Oyola SO, Rayner JC, Newbold CI, Berriman M, Spencer CC, McVean G, Day NP, White NJ, Bethell D, Dondorp AM, Plowe CV, Fairhurst RM, Kwiatkowski DP.** 2013. Multiple populations of artemisinin-resistant *Plasmodium falciparum* in Cambodia. Nat Genet **45:**648–655. http://dx.doi.org/10.1038/ng.2624.
3. **Olotu A, Fegan G, Wambua J, Nyangweso G, Awuondo KO, Leach A, Lievens M, Leboulleux D, Njuguna P, Peshu N, Marsh K, Bejon P.** 2013. Four-year efficacy of RTS,S/AS01E and its interaction with malaria exposure. N Engl J Med **368:**1111–1120. http://dx.doi.org/10.1056/NEJMoa1207564.
4. **Drinkwater N, McGowan S.** 2014. From crystal to compound: structure-based antimalarial drug discovery. Biochem J **461:**349–369. http://dx.doi.org/10.1042/BJ20140240.
5. **Spry C, van Schalkwyk DA, Strauss E, Saliba KJ.** 2010. Pantothenate utilization by *Plasmodium* as a target for antimalarial chemotherapy. Infect Disord Drug Targets **10:**200–216. http://dx.doi.org/10.2174/187152610791163390.
6. **Strauss E.** 2010. Coenzyme A biosynthesis and enzymology, p 351–410. *In* Mander L, Liu H-W (ed), Comprehensive natural products II: chemistry and biology, vol 7. Elsevier, Oxford, United Kingdom.
7. **Divo AA, Geary TG, Davis NL, Jensen JB.** 1985. Nutritional requirements of *Plasmodium falciparum* in culture. I. Exogenously supplied dialyzable components necessary for continuous growth. J Protozool **32:**59–64.
8. **Spry C, Kirk K, Saliba KJ.** 2008. Coenzyme A biosynthesis: an antimicrobial drug target. FEMS Microbiol Rev **32:**56–106. http://dx.doi.org/10.1111/j.1574-6976.2007.00093.x.
9. **Saliba K, Kirk K.** 2005. CJ-15,801, a fungal natural product, inhibits the intraerythrocytic stage of *Plasmodium falciparum* in vitro via an effect on pantothenic acid utilisation. Mol Biochem Parasitol **141:**129–131. http://dx.doi.org/10.1016/j.molbiopara.2005.02.003.
10. **Saliba KJ, Ferru I, Kirk K.** 2005. Provitamin B_5 (pantothenol) inhibits growth of the intraerythrocytic malaria parasite. Antimicrob Agents Chemother **49:**632–637. http://dx.doi.org/10.1128/AAC.49.2.632-637.2005.
11. **Spry C, Chai CL, Kirk K, Saliba KJ.** 2005. A class of pantothenic acid analogs inhibits *Plasmodium falciparum* pantothenate kinase and represses the proliferation of malaria parasites. Antimicrob Agents Chemother **49:**4649–4657. http://dx.doi.org/10.1128/AAC.49.11.4649-4657.2005.
12. **Fletcher S, Avery VM.** 2014. A novel approach for the discovery of chemically diverse anti-malarial compounds targeting the *Plasmodium falciparum* coenzyme A synthesis pathway. Malar J **13:**343. http://dx.doi.org/10.1186/1475-2875-13-343.
13. **Spry C, Macuamule C, Lin Z, Virga KG, Lee RE, Strauss E, Saliba KJ.** 2013. Pantothenamides are potent, on-target inhibitors of *Plasmodium falciparum* growth when serum pantetheinase is inactivated. PLoS One **8:**e54974. http://dx.doi.org/10.1371/journal.pone.0054974.
14. **van Schalkwyk DA, Chan XW, Misiano P, Gagliardi S, Farina C, Saliba KJ.** 2010. Inhibition of *Plasmodium falciparum* pH regulation by small molecule indole derivatives results in rapid parasite death. Biochem Pharmacol **79:**1291–1299. http://dx.doi.org/10.1016/j.bcp.2009.12.025.
15. **Sumanadasa SDM, Goodman CD, Lucke AJ, Skinner-Adams T, Sahama I, Haque A, Do TA, McFadden GI, Fairlie DP, Andrews KT.** 2012. Antimalarial activity of the anticancer histone deacetylase inhibitor SB939. Antimicrob Agents Chemother **56:**3849–3856. http://dx.doi.org/10.1128/AAC.00030-12.
16. **Kaskow BJ, Proffit JM, Blangero J, Moses EK, Abraham LJ.** 2012. Diverse biological activities of the vascular non-inflammatory molecules: the Vanin pantetheinases. Biochem Biophys Res Commun **417:**653–658. http://dx.doi.org/10.1016/j.bbrc.2011.11.099.
17. **Pace H, Brenner C.** 2001. The nitrilase superfamily: classification, structure and function. Genome Biol **2:**reviews0001.1–reviews0001.9. http://dx.doi.org/10.1186/gb-2001-2-1-reviews0001.
18. **Wittwer C, Wyse B, Hansen RG.** 1982. Assay of the enzymatic hydrolysis of pantetheine. Anal Biochem **122:**213–222. http://dx.doi.org/10.1016/0003-2697(82)90273-1.
19. **Wittwer CT, Burkhard D, Ririe K, Rasmussen R, Brown J, Wyse BW, Hansen RG.** 1983. Purification and properties of a pantetheine-hydrolyzing enzyme from pig kidney. J Biol Chem **258:**9733–9738.
20. **de Villiers M, Macuamule C, Spry C, Hyun Y-M, Strauss E, Saliba KJ.** 2013. Structural modification of pantothenamides counteracts degradation by pantetheinase and improves antiplasmodial activity. ACS Med Chem Lett **4:**784–789. http://dx.doi.org/10.1021/ml400180d.
21. **Hoegl A, Darabi H, Tran E, Awuah E, Kerdo ESC, Habib E, Saliba KJ, Auclair K.** 2014. Stereochemical modification of geminal dialkyl substituents on pantothenamides alters antimicrobial activity. Bioorg Med Chem Lett **24:**3274–3277. http://dx.doi.org/10.1016/j.bmcl.2014.06.013.
22. **Lipinski CA, Lombardo F, Dominy BW, Feeney PJ.** 2001. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv Drug Deliv Rev **46:**3–26. http://dx.doi.org/10.1016/S0169-409X(00)00129-0.