CHAPTER 4

This chapter consists of a full length text article to be submitted to *Antimicrobial Agents and Chemotherapy*. In this chapter the author guidelines are given, followed by the article prepared according these guidelines. The aims discussed in this chapter were:

1) to determine the *in vitro* efficacy of artemisone and its major active metabolite M1 on *P. falciparum* strains and also to evaluate the potential of the Pheroid® system to enhance the activity of artemisone
2) to determine if artemisone reference and metabolite M1 induce dormant parasites in the *P. falciparum* W2 strain and if the Pheroid® delivery system has an effect on the induction of dormancy.

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August 2013, Instructions to Authors, pages 1–22

2013 INSTRUCTIONS TO AUTHORS

SCOPE

Antimicrobial Agents and Chemotherapy (AAC) is an interdisciplinary journal devoted to the dissemination of knowledge relating to all aspects of antimicrobial and antiparasitic agents and chemotherapy. Within the circumscriptions set forth below, any report involving studies of or with antimicrobial, antiviral (including antiretroviral), antifungal, or antiparasitic agents as these relate to human disease is within the purview of AAC. Studies involving animal models, pharmacological characterization, and clinical trials are appropriate for consideration.

ASM publishes a number of different journals covering various aspects of the field of microbiology. Each journal has a prescribed scope that must be considered in determining the most appropriate journal for each manuscript. The following guidelines may be of assistance.

(i) Papers which describe the use of antimicrobial agents as tools for elucidating the basic biological processes of bacteria are considered more appropriate for the *Journal of Bacteriology*.

(ii) Manuscripts that (a) describe the use of antimicrobial or antiparasitic agents as tools in the isolation, identification, or epidemiology of microorganisms associated with disease; (b) are concerned with quality control procedures for diffusion, elution, or dilution tests for determining susceptibilities to antimicrobial agents in clinical laboratories; and (c) deal with applications of commercially prepared tests or kits to assays performed in clinical laboratories to measure the activities of established antimicrobial agents or their concentrations in body fluids are considered more appropriate for the *Journal of Clinical Microbiology*. Manuscripts concerned with the development or modification of assay methods (e.g., plasma antimicrobial concentrations and high-throughput screening techniques, etc.) and validation of their sensitivity and specificity with a sufficiently large number of determinations or compounds are considered appropriate for AAC.

(iii) Manuscripts describing new or novel methods or improvements in media and culture conditions will not be considered for publication in AAC unless these methods are applied to the study of problems related to the production or activity of antimicrobial agents. Such manuscripts are more appropriate for *Applied and Environmental Microbiology* or the *Journal of Clinical Microbiology*.

(iv) Manuscripts dealing with properties of unpurified natural products, with entities that are primarily antitumor agents, or with immunomodulatory agents that are not antimicrobial agents are not appropriate for AAC.

(v) Manuscripts dealing with novel small molecular antimicrobials must provide at least some data showing that the proposed new agents or scaffolds have the potential to become therapeutic agents. Appropriate demonstrations will vary but generally should be some combination of data on physical properties (solubility, protein binding, log *P* [logarithm of the ratio of the concentrations of un-ionized solute in solvents]), pharmacological properties (Caco2 predictions of bioavailability, pharmacokinetics in an animal species), or tolerability (mammalian cell toxicity, likelihood of hepatic metabolism, potential for receptor interactions, potential for human ERG liability). Initial presentations of compounds are not expected to address all these areas but rather to show an appropriate initial subset. For example, the first publication of a novel compound or compound series might address selected physical properties plus mammalian cell toxicity. Subsequent publications are expected to add progressively to the proof of the agent's therapeutic potential.

(vi) Biochemical analyses for β-lactamases that determine kinetic parameters (e.g., K_m, k_{cat}) must be performed on purified enzyme preparations. The enzyme must be in its native form, without any leader sequences or fusions used for purification (e.g., His tag). The determination of relative rates of hydrolysis may be performed on crude extracts.

(vii) Authors of papers describing enzymological studies should review the standards of the STRENDA Commission for information required for adequate description of experimental conditions and for reporting enzyme activity data (http://www.beilstein-institut.de/en/projekte/strenda/guidelines/).

(viii) A manuscript limited to the nucleic acid sequence of a gene encoding an antibiotic target, receptor, or resistance mechanism may be submitted as a Short-Form paper (see "Short-Form Papers") or a New-Data Letter to the Editor (see "Letters to the Editor"), depending on its length. Formatting instructions for nucleic acid sequences are given below (see "Presentation of Nucleic Acid Sequences"). Repetition of sequences already in a database should be avoided.

Questions about these guidelines may be directed to the editor in chief of the journal being considered.

If transfer to another ASM journal is recommended by an editor, the corresponding author will be contacted.

Note that a manuscript rejected by one ASM journal on scientific grounds or on the basis of its general suitability for publication is considered rejected by all other ASM journals.

EDITORIAL POLICY

Use of Microbiological Information

The Council Policy Committee (CPC) of the American Society for Microbiology affirms the long-standing position of the Society that microbiologists will work for the proper and beneficent application of science and will call to the attention of the public or the appropriate authorities misuses of microbiology or of information derived from microbiology. ASM members are obligated to discourage any use of microbiology contrary to the welfare of humankind, including the use of microbes as

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Ethical Guidelines

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Plagiarism is not limited to the text; it can involve any part of the manuscript, including figures and tables, in which material is copied from another publication without permission and attribution. An author may not reuse his or her own previously published work without attribution; this is considered self-plagiarism.

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Primary publication. Manuscripts submitted to the journal must represent reports of original research, and the original data must be available for review by the editor if necessary.

By submission of a manuscript to the journal, **the authors guarantee that they have the authority to publish the work and that the manuscript, or one with substantially the same content, was not published previously, is not being considered or published elsewhere, and was not rejected on scientific grounds by another ASM journal.** It is incumbent upon the author to acknowledge any prior publication, including his/her own articles, of the data contained in a manuscript submitted to an ASM journal. A copy of the relevant work should be submitted with the paper as supplemental material for information only. Whether the material constitutes the substance of a paper and therefore renders the manuscript unacceptable for publication is an editorial decision.

In brief, a paper is not acceptable for submission to an ASM journal if it, or its substance, has been made publicly available in:

- A serial, periodical, or book
- A conference report or symposium proceedings
- A technical bulletin or company white paper
- A public website
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- Posting of a method/protocol on a public website
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- Deposit of unpublished sequence data in a public database
- Preliminary disclosures of research findings as meeting posters, webcast as meeting presentations, or published in abstract form as adjuncts to a meeting, e.g., part of a program

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Patient Identification

When isolates are derived from patients in clinical studies, do not identify them by using the patients' initials, even as part of

a strain designation. Change the initials to numerals or use randomly chosen letters. Do not give hospital unit numbers; if a designation is needed, use only the last two digits of the unit. (Note: established designations of some viruses and cell lines, although they consist of initials, are acceptable [e.g., JC virus, BK virus, and HeLa cells].)

Nucleotide and Amino Acid Sequences

Newly determined nucleotide and/or amino acid sequence data must be deposited and GenBank/EMBL/DDBJ accession numbers must be included in the manuscript no later than the modification stage of the review process. It is expected that the sequence data will be released to the public no later than the publication (online posting) date of the accepted manuscript. The accession numbers should be included in a separate paragraph at the end of the Materials and Methods section for full-length papers or at the end of the text for Short-Form papers. If conclusions in a manuscript are based on the analysis of sequences and a GenBank/EMBL/DDBJ accession number is not provided at the time of the review, authors should provide the annotated sequence data as supplemental material for information only.

It is expected that, when previously published sequence accession numbers are cited in a manuscript, the original citations (e.g., journal articles) will be included in the References section when possible or reasonable.

Authors are also expected to do elementary searches and comparisons of nucleotide and amino acid sequences against the sequences in standard databases (e.g., GenBank) immediately before manuscripts are submitted and again at the proof stage.

Analyses should specify the database, and the date of each analysis should be indicated as, e.g., January 2013. If relevant, the version of the software used should be specified.

See "Presentation of Nucleic Acid Sequences" for nucleic acid sequence formatting instructions.

The URLs of the databases mentioned above are as follows: DNA Data Bank of Japan (DDBJ), http://www.ddbj.nig.ac.jp/; EMBL Nucleotide Sequence Database, http://www.ebi.ac.uk/ena/; and GenBank, National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/.

Proper Use of Locus Tags as Systematic Identifiers for Genes

To comply with recommendations from the International Nucleotide Sequence Database (INSD) Collaborators and to avoid conflicts in gene identification, researchers should implement the following two fundamental guidelines as standards for utilization of locus tags in genome analysis, annotation, submission, reporting, and publication. (i) Locus tag prefixes are systematic gene identifiers for all of the replicons of a genome and as such should be associated with a single genome project submission. (ii) New genome projects must be registered with the INSD, and new locus tag prefixes must be assigned in cooperation with the INSD to ensure that they conform to the agreed-upon criteria. Locus tag prefixes that are currently in use may be searched in the NCBI locus tag database (http://www.ncbi.nlm.nih.gov/genomes/lltp.cgi).

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Coordinates for new structures of macromolecules determined by X-ray crystallography or cryo-electron microscopy must be deposited in the Protein Data Bank and assigned identification codes must be included in the manuscript no later than the modification stage of the review process. It is expected that the coordinates will be released to the public no later than the publication (online posting) date of the accepted manuscript. Authors are encouraged to send coordinates with their original submission, however, so that reviewers can examine them along with the manuscript. The accession number(s) should be listed in a separate paragraph at the end of the Materials and Methods section for full-length papers or at the end of the text for Short-Form papers.

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The URLs of the databases mentioned above are as follows: Gene Expression Omnibus (GEO), http://www.ncbi.nlm.nih.gov/geo/; ArrayExpress, http://www.ebi.ac.uk/arrayexpress/; and Center for Information Biology Gene Expression Database (CIBEX), http://cibex.nig.ac.jp/data/index.html.

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AAC expects authors to deposit strains used in therapeutic activity assessments and studies of mechanisms of action, resistance, and cross-resistance in publicly accessible culture collections and to refer to the collections and strain numbers in the text. Since the authenticity of subcultures of culture collection specimens that are distributed by individuals cannot be ensured, authors should indicate laboratory strain designations and donor sources as well as original culture collection identification numbers.

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New scientific names of fungi along with key nomenclatural and descriptive material must be deposited in MycoBank (http://www.mycobank.org/) and the assigned accession number(s) must be included in the manuscript no later than the modification stage of the review process. It is expected that the data will be released to the public no later than the publication (online posting) date of the accepted manuscript. Authors are

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Supplemental material will be peer reviewed along with the manuscript and must be uploaded to the eJournalPress (eJP) peer review system at initial manuscript submission. The decision to publish the material online with the accepted article is made by the editor. It is possible that a manuscript will be accepted but that the supplemental material will not be.

The number of supplemental material files is limited to 10. Supplemental files should be submitted in the following standard formats.

- **Text, figures, tables, and legends** should be included in a single PDF file. All figures and tables should be numbered independently and cited at the relevant point in the manuscript text, e.g. "Fig. S1," "Fig. S2," "Table S3," etc. Do not duplicate data by presenting them in both the text and the figure. Each legend should appear below its corresponding figure or table. The maximum file size is 8 MB. Please review this sample file for guidance.
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Manuscripts that have been rejected, or withdrawn after being returned for modification, may be resubmitted to the same ASM journal if the major criticisms have been addressed. A manuscript rejected by one ASM journal on scientific grounds or on the basis of its general suitability for publication is considered rejected by all other ASM journals; however, a manuscript rejected solely on the basis of scope may be "resubmitted" to a more appropriate ASM journal.

For all resubmissions (to the same or a different journal, irrespective of the extent of the revisions and irrespective of the amount of time between rejection and resubmission), the cover letter must state that the manuscript is a resubmission, and the former manuscript control number must be provided. A point-by-point response to the review(s) must be loaded as a separate file (identified as such), and a compare copy of the revised manuscript showing the changes must be included as a Marked Up Manuscript. Manuscripts resubmitted to the same journal are normally handled by the original editor.

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2. **Falagas ME, Kasiakou SK.** 2006. Use of international units when dosing colistin will help decrease confusion related to various formulations of the drug around the world. Antimicrob. Agents Chemother. **50:**2274–2275. (Letter.) {*"Letter" or "Letter to the editor" is allowed but not required at the end of such an entry.*}
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4. **da Costa MS, Nobre MF, Rainey FA.** 2001. Genus I. Thermus Brock and Freeze 1969, 295,[AL] emend. Nobre, Trüper and da Costa 1996b, 605, p 404–414. *In* Boone DR, Castenholz RW, Garrity GM (ed), Bergey's manual of systematic bacteriology, 2nd ed, vol 1. Springer, New York, NY.
5. **Stratagene.** 2006. Yeast DNA isolation system: instruction manual. Stratagene, La Jolla, CA. {*Use the company name as the author if none is provided for a company publication.*}

6. **Forman MS, Valsamakis A.** 2011. Specimen collection, transport, and processing: virology, p 1276–1288. *In* Versalovic J, Carroll KC, Jorgensen JH, Funke G, Landry ML, Warnock DW (ed), Manual of clinical microbiology, 10th ed, vol 2. ASM Press, Washington, DC.
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8. **García CO, Paira S, Burgos R, Molina J, Molina JF, Calvo C, Vega L, Jara LJ, García-Kutzbach A, Cuellar ML, Espinoza LR.** 1996. Detection of *Salmonella* DNA in synovial membrane and synovial fluid from Latin American patients using the polymerase chain reaction. Arthritis Rheum. **39**(Suppl 9):S185. {*Meeting abstract published in journal supplement.*}
9. **Smith D, Johnson C, Maier M, Maurer JJ.** 2005. Distribution of fimbrial, phage and plasmid associated virulence genes among poultry *Salmonella enterica* serovars, abstr P-038, p 445. Abstr. 105th Gen. Meet. Am. Soc. Microbiol. American Society for Microbiology, Washington, DC. {*Abstract title is optional.*}
10. **Rotimi VO, Salako NO, Mohaddas EM, Philip LP.** 2005. Abstr. 45th Intersci. Conf. Antimicrob. Agents Chemother., abstr D-1658. {*Abstract title is optional.*}
11. **Green PN, Hood D, Dow CS.** 1984. Taxonomic status of some methylotrophic bacteria, p 251–254. *In* Crawford RL, Hanson RS (ed), Microbial growth on C_1 compounds. Proceedings of the 4th International Symposium. American Society for Microbiology, Washington, DC.
12. **O'Malley DR.** 1998. Ph.D. thesis. University of California, Los Angeles, CA. {*Title is optional.*}
13. **Odell JC.** April 1970. Process for batch culturing. US patent 484,363,770. {*Include the name of the patented item/process if possible; the patent number is mandatory.*}
14. **Elder BL, Sharp SE.** 2003. Cumitech 39, Competency assessment in the clinical laboratory. Coordinating ed, Sharp SE. ASM Press, Washington, DC.

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3. **Dionne MS, Schneider DS.** 2002. Screening the fruitfly immune system. Genome Biol. 3:REVIEWS1010. http://genomebiology.com/2002/3/4/reviews/1010.
4. **Gregory ST.** 2 September 2009, posting date. Chapter 2.5.4, Structural basis for the decoding mechanism. *In* Böck A, et al (ed), EcoSal—*Escherichia coli* and *Salmonella*: cellular and molecular biology. ASM Press, Washington, DC. doi: 10.1128/ecosal.2.5.4. {*Note that each chapter has its own posting date.*}

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. . . as described previously (M. G. Gordon and F. L. Rattner, presented at the Fourth Symposium on Food Microbiology, Overton, IL, 13 to 15 June 1989). {*For nonpublished abstracts and posters, etc.*}
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Retractions

Retractions are reserved for major errors or breaches of ethics that, for example, may call into question the source of the data or the validity of the results and conclusions of an article. Submit Retractions via the eJP online manuscript submission and peer review system (see "Submission, Review, and Publication Processes"). In the Abstract section of the submission form (a required field), put "Not Applicable." Upload the text of your Retraction as a Microsoft Word file. Letters of agreement signed by all of the authors must be supplied as supplemental material for information only (scanned PDF files). The Retraction will be assigned to the editor in chief of the journal, and the editor who handled the paper and the chairperson of the ASM Publications Board will be consulted. If all parties agree to the publication and content of the Retraction, it will be sent to the Journals Department for publication.

ILLUSTRATIONS AND TABLES

Illustrations

Image manipulation. Computer-generated images may be processed only minimally. Processing (e.g., changing contrast, brightness, or color balance) is acceptable only if applied to all parts of the image, as well as to the controls, equally, and descriptions of all such adjustments and the tools used (both hardware and software) must be provided in the manuscript. Also, ASM considers unacknowledged spliced images to be data manipulation and therefore a potential ethical violation. Unprocessed data and files must be retained by the authors and be provided to the editor on request.

File types and formats. Illustrations may be continuous-tone images, line drawings, or composites. Color graphics may be submitted, but the cost of printing in color must be borne by the author. Suggestions about how to reduce costs and ensure accurate color reproduction are given below.

On initial submission, illustrations should be supplied as PDF files, with the legend on the same page, to assist review. At the modification stage, production quality digital files must be provided, and the legends should appear in the manuscript text file. The legends are copyedited and typeset for final publication, not included as part of the figure itself. All graphics submitted with modified manuscripts must be bitmap, grayscale, or in the RGB (preferred) or CMYK color mode. See "Color illustrations." Halftone images (those with various densities or shades) must be grayscale, not bitmap. AAC accepts TIFF or EPS files but discourages PowerPoint for either black-and-white or color images.

For instructions on creating acceptable EPS and TIFF files, refer to the Cadmus digital art website, http://art.cadmus.com/da/index.jsp. PowerPoint requires users to pay close attention to the fonts used in their images (see the section on fonts below). If instructions for fonts are not followed exactly, images prepared for publication are subject to missing characters, improperly converted characters, or shifting/obscuring of elements or text in the figure. For proper font use in PowerPoint images, refer to the Cadmus digital art website, http://art.cadmus.com/da/instructions/ppt_disclaimer.jsp.

We strongly recommend that before returning their modified manuscripts, authors check the acceptability of their digital images for production by running their files through Rapid Inspector, a tool provided at the following URL: http://rapidinspector.cadmus.com/RapidInspector/zmw/index.jsp. Rapid Inspector is an easy-to-use, Web-based application that

identifies file characteristics that may render the image unusable for production.

If you have additional questions about using the Rapid Inspector preflighting tool, please send an e-mail inquiry to digitalart@cadmus.com.

Minimum resolution. It is extremely important that a high enough file resolution is used. All separate images that you import into a figure file must be at the correct resolution before they are placed. (For instance, placing a 72-dpi image in a 300-dpi EPS file will not result in the placed image meeting the minimum requirements for file resolution.) Note, however, that the higher the resolution, the larger the file and the longer the upload time. Publication quality will not be improved by using a resolution higher than the minimum. Minimum resolutions are as follows:

300 dpi for grayscale and color
600 dpi for combination art (lettering and images)
1,200 dpi for line art

Size. All graphics **should be submitted at their intended publication size;** that is, the image uploaded should be 100% of its print dimensions so that no reduction or enlargement is necessary. Resolution must be at the required level at the submitted size. Include only the significant portion of an illustration. White space must be cropped from the image, and excess space between panel labels and the image must be eliminated.

Maximum width for a 1-column figure: 20.6 picas (ca. 8.7 cm)
Maximum width for a 2-column figure: 42 picas (ca. 17.8 cm)
Minimum width for a 2-column figure: 26 picas (11.1 cm)
Maximum height for a standard figure: 54.7 picas (ca. 23.2 cm)
Maximum height for an oversized figure (no running title): 57.4 picas (ca. 24.3 cm)

Contrast. Illustrations must contain sufficient contrast to be viewed easily on a monitor or on the printed page.

Labeling and assembly. All final lettering and labeling must be incorporated into the figures. On initial submission, illustrations should be provided as PDF files, with the legend beneath each image, to assist review. At the modification stage, production quality digital figure files must be provided, and the legends should appear in the manuscript text file. Put the figure number well outside the boundaries of the image itself. (Numbering may need to be changed at the copyediting stage.) Each figure must be uploaded as a separate file, and any multipanel figures must be assembled into one file; i.e., rather than uploading a separate file for each panel in a figure, assemble all panels in one piece and supply them as one file.

Fonts. To avoid font problems, set all type in one of the following fonts: Arial, Helvetica, Times Roman, European PI, Mathematical PI, or Symbol. Courier may be used but should be limited to nucleotide or amino acid sequences in which a nonproportional (monospace) font is required. All fonts other than these must be converted to paths (or outlines) in the application with which they were created.

Color illustrations. Color costs must be borne by the author. See "Publication Fees." All figures submitted in color will be processed as color. Adherence to the following guidelines will help to minimize costs and to ensure color reproduction that is as accurate as possible.

The final online version is considered the version of record for AAC and all other ASM journals. To maximize online reproduction, color illustrations should be supplied in the RGB color mode as either (i) RGB TIFF images with a resolution of at least 300 pixels per inch (raster files, consisting of pixels) or (ii) Illustrator-compatible EPS files with RGB color elements (vector files, consisting of lines, fonts, fills, and images). CMYK files are also accepted. Other than in color space, CMYK files must meet the same production criteria as RGB files. The RGB color space is the native color space of computer monitors and of most of the equipment and software used to capture scientific data, and it can display a wider range of colors (especially bright fluorescent hues) than the CMYK (cyan, magenta, yellow, black) color space used by print devices that put ink (or toner) on paper. For the print version (and reprints), ASM's print provider will automatically create CMYK versions of color illustrations from the supplied RGB versions. Color in the print journal may not match that in the online journal of record because of the smaller range of colors capable of being reproduced by CMYK inks on a printing press. For additional information on RGB versus CMYK color, refer to the Cadmus digital art site, http://art.cadmus.com/da/guidelines_rgb.jsp.

Drawings

Submit graphs, charts, complicated chemical or mathematical formulas, diagrams, and other drawings as finished products not requiring additional artwork or typesetting. All elements, including letters, numbers, and symbols, must be easily readable, and both axes of a graph must be labeled. Keep in mind that the journal is published both in print and online and that the same electronic files submitted by the authors are used to produce both.

When creating line art, please use the following guidelines:

(i) **All art must be submitted at its intended publication size.** For acceptable dimensions, see "Size," above.

(ii) **Avoid using screens (i.e., shading) in line art.** It can be difficult and time-consuming to reproduce these images without moiré patterns. Various pattern backgrounds are preferable to screens as long as the patterns are not imported from another application. If you must use images containing screens,

(a) Generate the image at line screens of 85 lines per inch or less.

(b) When applying multiple shades of gray, differentiate the gray levels by at least 20%.

(c) Never use levels of gray below 5% or above 95% as they are likely to fade out or become totally black when output.

(iii) Use thick, solid lines that are no finer than 1 point in thickness.

(iv) No type should be smaller than 6 points at the final publication size.

(v) Avoid layering type directly over shaded or textured areas.

(vi) Avoid the use of reversed type (white lettering on a black background).

(vii) Avoid heavy letters, which tend to close up, and unusual symbols, which the printer may not be able to reproduce in the legend.

(viii) If colors are used, avoid using similar shades of the same color and avoid very light colors.

In figure ordinate and abscissa scales (as well as table column headings), avoid the ambiguous use of numbers with exponents. Usually, it is preferable to use the Système International d'Unités (SI) symbols (μ for 10^{-6}, m for 10^{-3}, k for 10^{3}, and M for 10^{6}, etc.). Thus, a representation of 20,000 cpm on a figure ordinate should be made by the number 20 accompanied by the label kcpm. A complete listing of SI symbols can be found in the International Union of Pure and Applied Chemistry (IUPAC) publication *Quantities, Units and Symbols in Physical Chemistry* (RSC Publishing, Cambridge, United Kingdom, 2007); an abbreviated list is available at http://old.iupac.org/reports/1993/homann/index.html.

When powers of 10 must be used, the journal requires that the exponent power be associated with the number shown. In representing 20,000 cells per ml, the numeral on the ordinate should be "2" and the label should be "10^{4} cells per ml" (not "cells per ml $\times$ 10^{-4}"). Likewise, an enzyme activity of 0.06 U/ml might be shown as 6 accompanied by the label "10^{-2} U/ml." The preferred designation is 60 mU/ml (milliunits per milliliter).

Presentation of Nucleic Acid Sequences

Long nucleic acid sequences must be presented as figures in the following format to conserve space. Print the sequence in lines of approximately 100 to 120 nucleotides in a nonproportional (monospace) font that is easily legible when published with a line length of 6 inches (ca. 15.2 cm). If possible, lines of nucleic acid sequence should be further subdivided into blocks of 10 or 20 nucleotides by spaces within the sequence or by marks above it. Uppercase and lowercase letters may be used to designate the exon-intron structure or transcribed regions, etc., if the lowercase letters remain legible at a 6-inch (ca. 15.2-cm) line length. Number the sequence line by line; place numerals representing the first base of each line to the left of the lines. Minimize spacing between lines of sequence, leaving room only for annotation of the sequence. Annotation may include boldface, underlining, brackets, and boxes, etc. Encoded amino acid sequences may be presented, if necessary, immediately above or below the first nucleotide of each codon, by using the single-letter amino acid symbols. Comparisons of multiple nucleic acid sequences should conform as nearly as possible to the same format.

TABLE 1 Distribution of protein and ATPase in fractions of dialyzed membranes[a]

Membrane	Fraction	ATPase	
		U/mg of protein	Total U
Control	Depleted membrane	0.036	2.3
	Concentrated supernatant	0.134	4.82
EI treated	Depleted membrane	0.034	1.98
	Concentrated supernatant	0.11	4.6

[a] Specific activities of ATPase of nondepleted membranes from control and treated bacteria were 0.21 and 0.20, respectively.

Figure Legends

On initial submission, to assist review, the legend should be incorporated in the image file and appear beneath the figure. At the modification stage, figure legends must instead appear in the manuscript text file.

Legends should provide enough information so that the figure is understandable without frequent reference to the text. However, detailed experimental methods must be described in the Materials and Methods section, not in a figure legend. A method that is unique to one of several experiments may be set forth in a legend only if the description is very brief (one or two sentences). Define all symbols used in the figure and define all abbreviations that are not used in the text.

Tables

Tables that contain artwork, chemical structures, or shading must be submitted as illustrations in an acceptable format at the modification stage. The preferred format for regular tables is Microsoft Word; however, WordPerfect and Acrobat PDF are also acceptable. Note that a straight Excel file is not currently an acceptable format. Excel files must be either embedded in a Word or WordPerfect document or converted to PDF before being uploaded.

Tables should be formatted as follows. Arrange the data so that **columns of like material read down, not across.** The headings should be sufficiently clear so that the meaning of the data is understandable without reference to the text. See the "Abbreviations" section of these Instructions for those that should be used in tables. Explanatory footnotes are acceptable, but more-extensive table "legends" are not. Footnotes should not include detailed descriptions of the experiment. Tables must include enough information to warrant table format; those with fewer than six pieces of data will be incorporated into the text by the copy editor. Table 1 is an example of a well-constructed table.

Avoid tables (or figures) of raw data on drug susceptibility, therapeutic activity, or toxicity. Such data should be analyzed

by an approved procedure, and the results should be presented in tabular form.

NOMENCLATURE

Chemical and Biochemical Nomenclature

The recognized authority for the names of chemical compounds is *Chemical Abstracts* (CAS; http://www.cas.org/) and its indexes. *The Merck Index*, 14th ed. (Merck & Co., Inc., Whitehouse Station, NJ, 2006), is also an excellent source. For guidelines to the use of biochemical terminology, consult *Biochemical Nomenclature and Related Documents* (Portland Press, London, United Kingdom, 1992), available at http://www.chem.qmul.ac.uk/iupac/bibliog/white.html, and the instructions to authors of the *Journal of Biological Chemistry* and the *Archives of Biochemistry and Biophysics*.

Molecular weight should not be expressed in daltons; molecular weight is a unitless ratio. Molecular mass is expressed in daltons.

For enzymes, use the recommended (trivial) name as assigned by the Nomenclature Committee of the International Union of Biochemistry (IUB) as described in *Enzyme Nomenclature* (Academic Press, Inc., New York, NY, 1992) and its supplements and at http://www.chem.qmul.ac.uk/iubmb/enzyme/. If a nonrecommended name is used, place the proper (trivial) name in parentheses at first use in the abstract and text. Use the EC number when one has been assigned. Authors of papers describing enzymological studies should review the standards of the STRENDA Commission for information required for adequate description of experimental conditions and for reporting enzyme activity data (http://www.beilstein-institut.de/en/projekte/strenda/guidelines/).

Nomenclature of Microorganisms

Binary names, consisting of a generic name and a specific epithet (e.g., *Escherichia coli*), must be used for all microorganisms. Names of categories at or above the genus level may be used alone, but specific and subspecific epithets may not. A specific epithet must be preceded by a generic name, written out in full the first time it is used in a paper. Thereafter, the generic name should be abbreviated to the initial capital letter (e.g., *E. coli*), provided there can be no confusion with other genera used in the paper. Names of all taxa (kingdoms, phyla, classes, orders, families, genera, species, and subspecies) are printed in italics and should be italicized in the manuscript; strain designations and numbers are not. Vernacular (common) names should be in lowercase roman type (e.g., streptococcus, brucella). For *Salmonella*, genus, species, and subspecies names should be rendered in standard form: *Salmonella enterica* at first use, *S. enterica* thereafter; *Salmonella enterica* subsp. *arizonae* at first use, *S. enterica* subsp. *arizonae* thereafter. Names of serovars should be in roman type with the first letter capitalized: *Salmonella enterica* serovar Typhimurium. After the first use, the serovar may also be given without a species name: *Salmonella* Typhimurium, *S.* Typhimurium, or *Salmonella* serovar Typhimurium. For other information regarding serovar designations, see *Antigenic Formulae of the Salmonella Serovars*, 9th ed. (P. A. D. Grimont and F.-X. Weill, WHO Collaborating Centre for Reference and Research on Salmonella, Institut Pasteur, Paris, France, 2007; see http://www.pasteur.fr/ip/portal/action/WebdriveActionEvent/oid/01s-000036-089). For a summary of the current standards for *Salmonella* nomenclature and the Kaufmann-White criteria, see the article by Brenner et al. (J. Clin. Microbiol. 38:2465–2467, 2000), the opinion of the Judicial Commission of the International Committee on Systematics of Prokaryotes (Int. J. Syst. Evol. Microbiol. 55:519–520, 2005), and the article by Tindall et al. (Int. J. Syst. Evol. Microbiol. 55:521–524, 2005).

The spelling of bacterial names should follow the *Approved Lists of Bacterial Names (Amended) & Index of the Bacterial and Yeast Nomenclatural Changes* (V. B. D. Skerman et al., ed., American Society for Microbiology, Washington, DC, 1989) and the validation lists and notification lists published in the *International Journal of Systematic and Evolutionary Microbiology* (formerly the *International Journal of Systematic Bacteriology*) since January 1989. In addition, two sites on the World Wide Web list current approved bacterial names: Bacterial Nomenclature Up-to-Date (http://www.dsmz.de/bacterial-diversity/prokaryotic-nomenclature-up-to-date.html) and List of Prokaryotic Names with Standing in Nomenclature (http://www.bacterio.cict.fr/). If there is reason to use a name that does not have standing in nomenclature, the name should be enclosed in quotation marks in the title and at its first use in the abstract and the text and an appropriate statement concerning the nomenclatural status of the name should be made in the text. "*Candidatus*" species should always be set in quotation marks.

Since the classification of fungi is not complete, it is the responsibility of the author to determine the accepted binomial for a given organism. Sources for these names include *The Yeasts: a Taxonomic Study*, 5th ed. (C. P. Kurtzman, J. W. Fell, and T. Boekhout, ed., Elsevier Science, Amsterdam, Netherlands, 2011), and *Dictionary of the Fungi*, 10th ed. (P. M. Kirk, P. F. Cannon, D. W. Minter, and J. A. Stalpers, ed., CABI International, Wallingford, Oxfordshire, United Kingdom, 2008); see also http://www.speciesfungorum.org/Names/Fundic.asp.

Names used for viruses should be those approved by the International Committee on Taxonomy of Viruses (ICTV) and reported on the ICTV Virus Taxonomy website (http://www.ictvonline.org/index.asp). In addition, the recommendations of the ICTV regarding the use of species names should generally be followed: when the entire species is discussed as a taxonomic entity, the species name, as with other taxa, is italic and has the first letter and any proper nouns capitalized (e.g., *Tobacco mosaic virus*, *Murray Valley encephalitis virus*). When the behavior or manipulation of individual viruses is discussed, the vernacular (e.g., tobacco mosaic virus, Murray Valley encephalitis virus) should be used. If desired, synonyms may be added parenthetically when the name is first mentioned. Approved generic (or group) and family names may also be used.

Microorganisms, viruses, and plasmids should be given designations consisting of letters and serial numbers. It is generally advisable to include a worker's initials or a descriptive symbol of locale or laboratory, etc., in the designation. Each new strain, mutant, isolate, or derivative should be given a new

(serial) designation. This designation should be distinct from those of the genotype and phenotype, and genotypic and phenotypic symbols should not be included. Plasmids are named with a lowercase "p" followed by the designation in uppercase letters and numbers. To avoid the use of the same designation as that of a widely used strain or plasmid, check the designation against a publication database such as Medline.

Genetic Nomenclature

To facilitate accurate communication, **it is important that standard genetic nomenclature be used whenever possible and that deviations or proposals for new naming systems be endorsed by an appropriate authoritative body**. Review and/or publication of submitted manuscripts that contain new or nonstandard nomenclature may be delayed by the editor or the Journals Department so that they may be reviewed.

Bacteria. The genetic properties of bacteria are described in terms of phenotypes and genotypes. The phenotype describes the observable properties of an organism. The genotype refers to the genetic constitution of an organism, usually in reference to some standard wild type. The guidelines that follow are based on the recommendations of Demerec et al. (Genetics 54:61–76, 1966).

(i) Phenotype designations must be used when mutant loci have not been identified or mapped. They can also be used to identify the protein product of a gene, e.g., the OmpA protein. Phenotype designations generally consist of three-letter symbols; these are not italicized, and the first letter of the symbol is capitalized. It is preferable to use Roman or Arabic numerals (instead of letters) to identify a series of related phenotypes. Thus, a series of nucleic acid polymerase mutants might be designated Pol1, Pol2, and Pol3, etc. Wild-type characteristics can be designated with a superscript plus (Pol^+), and, when necessary for clarity, negative superscripts (Pol^-) can be used to designate mutant characteristics. Lowercase superscript letters may be used to further delineate phenotypes (e.g., Str^r for streptomycin resistance). Phenotype designations should be defined.

(ii) Genotype designations are also indicated by three-letter locus symbols. In contrast to phenotype designations, these are lowercase italic (e.g., *ara his rps*). If several loci govern related functions, these are distinguished by italicized capital letters following the locus symbol (e.g., *araA araB araC*). Promoter, terminator, and operator sites should be indicated as described by Bachmann and Low (Microbiol. Rev. 44:1–56, 1980): e.g., *lacZp*, *lacAt*, and *lacZo*.

(iii) Wild-type alleles are indicated with a superscript plus (*ara*$^+$ *his*$^+$). A superscript minus is not used to indicate a mutant locus; thus, one refers to an *ara* mutant rather than an *ara*$^-$ strain.

(iv) Mutation sites are designated by placing serial isolation numbers (allele numbers) after the locus symbol (e.g., *araA1 araA2*). If only a single such locus exists or if it is not known in which of several related loci the mutation has occurred, a hyphen is used instead of the capital letter (e.g., *ara-23*). It is essential in papers reporting the isolation of new mutants that allele numbers be given to the mutations. For *Escherichia coli*, there is a registry of such numbers: *E. coli* Genetic Stock Center (http://cgsc.biology.yale.edu/). For the genus *Salmonella*, the registry is *Salmonella* Genetic Stock Center (http://people.ucalgary.ca/~kesander/). For the genus *Bacillus*, the registry is *Bacillus* Genetic Stock Center (http://www.bgsc.org/).

(v) The use of superscripts with genotypes (other than + to indicate wild-type alleles) should be avoided. Designations indicating amber mutations (Am), temperature-sensitive mutations (Ts), constitutive mutations (Con), cold-sensitive mutations (Cs), production of a hybrid protein (Hyb), and other important phenotypic properties should follow the allele number [e.g., *araA230*(Am) *hisD21*(Ts)]. All other such designations of phenotype must be defined at the first occurrence. If superscripts must be used, they must be approved by the editor and defined at the first occurrence in the text.

Subscripts may be used in two situations. Subscripts may be used to distinguish between genes (having the same name) from different organisms or strains; e.g., $his_{E.\ coli}$ or $his_{K\text{-}12}$ for the *his* gene of *E. coli* or strain K-12, respectively, may be used to distinguish this gene from the *his* gene in another species or strain. An abbreviation may also be used if it is explained. Similarly, a subscript is also used to distinguish between genetic elements that have the same name. For example, the promoters of the *gln* operon can be designated $glnAp_1$ and $glnAp_2$. This form departs slightly from that recommended by Bachmann and Low (e.g., *desC1p*).

(vi) Deletions are indicated by the symbol Δ placed before the deleted gene or region, e.g., Δ*trpA432*, Δ(*aroP-aceE*)*419*, or Δ(*hisQ-hisJo*)*1256*. Similarly, other symbols can be used (with appropriate definition). Thus, a fusion of the *ara* and *lac* operons can be shown as Φ(*ara-lac*)95. Likewise, Φ(*araB'-lacZ*$^+$)*96* indicates that the fusion results in a truncated *araB* gene fused to an intact *lacZ* gene, and Φ(*malE-lacZ*)*97*(Hyb) shows that a hybrid protein is synthesized. An inversion is shown as IN(*rrnD-rrnE*)*1*. An insertion of an *E. coli his* gene into plasmid pSC101 at zero kilobases (0 kb) is shown as pSC101 Ω(0kb::K-12*hisB*)*4*. An alternative designation of an insertion can be used in simple cases, e.g., *galT236*::Tn*5*. The number *236* refers to the locus of the insertion, and if the strain carries an additional *gal* mutation, it is listed separately. Additional examples, which utilize a slightly different format, can be found in the papers by Campbell et al. and Novick et al. cited below. It is important in reporting the construction of strains in which a mobile element was inserted and subsequently deleted that this fact be noted in the strain table. This can be done by listing the genotype of the strain used as an intermediate in a table footnote or by making a direct or parenthetical remark in the genotype, e.g., (F^-), ΔMu *cts*, or *mal*::ΔMu *cts*::*lac*. In setting parenthetical remarks within the genotype or dividing the genotype into constituent elements, parentheses and brackets are used without special meaning; brackets are used outside parentheses. To indicate the presence of an episome, parentheses (or brackets) are used (λ, F^+). Reference to an integrated episome is indicated as described above for inserted elements, and an exogenote is shown as, for example, W3110/F'8(*gal*$^+$).

For information about genetic maps of locus symbols in current use, consult Berlyn (Microbiol. Mol. Biol. Rev. 62: 814–984, 1998) for *E. coli* K-12, Sanderson and Roth (Micro-

biol. Rev. **52**:485–532, 1988) for *Salmonella* serovar Typhimurium, Holloway et al. (Microbiol. Rev. **43**:73–102, 1979) for the genus *Pseudomonas*, Piggot and Hoch (Microbiol. Rev. **49**: 158–179, 1985) for *Bacillus subtilis*, Perkins et al. (Microbiol. Rev. **46**:426–570, 1982) for *Neurospora crassa*, and Mortimer and Schild (Microbiol. Rev. **49**:181–213, 1985) for *Saccharomyces cerevisiae*. For yeasts, *Chlamydomonas* spp., and several fungal species, symbols such as those given in the *Handbook of Microbiology*, 2nd ed. (A. I. Laskin and H. A. Lechevalier, ed., CRC Press, Inc., Cleveland, OH, 1988) should be used.

Conventions for naming genes. It is recommended that (entirely) new genes be given names that are mnemonics of their function, avoiding names that are already assigned and earlier or alternative gene names, irrespective of the bacterium for which such assignments have been made. Similarly, it is recommended that, whenever possible, orthologous genes present in different organisms receive the same name. When homology is not apparent or the function of a new gene has not been established, a provisional name may be given by one of the following methods. (i) The gene may be named on the basis of its map location in the style *yaaA*, analogous to the style used for recording transposon insertions (*zef*) as discussed below. A list of such names in use for *E. coli* has been published by Rudd (Microbiol. Mol. Biol. Rev. **62**:985–1019, 1998). (ii) A provisional name may be given in the style described by Demerec et al. (e.g., *usg*, gene upstream of *folC*). Such names should be unique, and names such as *orf* or *genX* should not be used. For reference, the *E. coli* Genetic Stock Center's database includes an updated listing of *E. coli* gene names and gene products. It is accessible on the Internet (http://cgsc.biology.yale.edu/index.php). A list can also be found in the work of Riley (Microbiol. Rev. **57**:862–952, 1993). For the genes of other bacteria, consult the references given above.

For prokaryotes, gene names should not begin with prefixes indicating the genus and species from which the gene is derived. (However, subscripts may be used where necessary to distinguish between genes from different organisms or strains as described in section v of "Bacteria," above.) For eukaryotes, such prefixes may be used for clarity when discussing genes with the same name from two different organisms (e.g., Sc*URA3* versus Ca*URA3*); the prefixes are not considered part of the gene name proper and are not italicized.

Locus tags. Locus tags are systematic, unique identifiers that are assigned to each gene in GenBank. All genes mentioned in a manuscript should be traceable to their sequences by the reader, and locus tags may be used for this purpose in manuscripts to identify uncharacterized genes. In addition, authors should check GenBank to make sure that they are using the correct, up-to-date format for locus tags (e.g., uppercase versus lowercase letters and the presence or absence of an underscore, etc.). Locus tag formats vary between different organisms and also may be updated for a given organism, so it is important to check GenBank at the time of manuscript preparation.

"Mutant" versus "mutation." Keep in mind the distinction between a mutation (an alteration of the primary sequence of the genetic material) and a mutant (a strain carrying one or more mutations). One may speak about the mapping of a mutation, but one cannot map a mutant. Likewise, a mutant has no genetic locus, only a phenotype.

"Homology" versus "similarity." For use of terms that describe relationships between genes, consult the articles by Theissen (Nature **415**:741, 2002) and Fitch (Trends Genet. **16**: 227–231, 2000). "Homology" implies a relationship between genes that have a common evolutionary origin; partial homology is not recognized. When sequence comparisons are discussed, it is more appropriate to use the term "percent sequence similarity" or "percent sequence identity," as appropriate.

Strain designations. Do not use a genotype as a name (e.g., ". . . subsequent use of *leuC6* for transduction . . ."). If a strain designation has not been chosen, select an appropriate word combination (e.g., "another strain containing the *leuC6* mutation").

Viruses. The genetic nomenclature for viruses differs from that for bacteria. In most instances, viruses have no phenotype, since they have no metabolism outside host cells. Therefore, distinctions between phenotype and genotype cannot be made. Superscripts are used to indicate hybrid genomes. Genetic symbols may be one, two, or three letters. For example, a mutant strain of λ might be designated λ *Aam11 int2 red114 cI857*; this strain carries mutations in genes *cI*, *int*, and *red* and an amber-suppressible (am) mutation in gene *A*. A strain designated λ att^{434} imm^{21} would represent a hybrid of phage λ that carries the immunity region (*imm*) of phage 21 and the attachment (*att*) region of phage 434. Host DNA insertions into viruses should be delineated by square brackets, and the genetic symbols and designations for such inserted DNA should conform to those used for the host genome. Genetic symbols for phage λ can be found in reports by Szybalski and Szybalski (Gene **7**:217–270, 1979) and Echols and Murialdo (Microbiol. Rev. **42**:577–591, 1978).

Eukaryotes. FlyBase (http://flybase.org/) is the genetic nomenclature authority for *Drosophila melanogaster*. WormBase (http://wormbase.org/#01-23-6) is the genetic nomenclature authority for *Caenorhabditis elegans*. When naming genes for *Aspergillus* species, the nomenclature guidelines posted at http://www.aspergillus.org.uk/indexhome.htm?secure/sequence_info/nomenclature.htm should be followed, and the *Aspergillus* Genome Database (http://www.aspgd.org/) should be searched to ensure that any new name is not already in use. The *Saccharomyces* Genome Database (http://www.yeastgenome.org/) and the *Candida* Genome Database (http://www.candidagenome.org/) are authorities for *Saccharomyces cerevisiae* and *Candida albicans* genetic nomenclature, respectively. For information about the genetic nomenclature of other eukaryotes, see the Instructions to Authors for *Eukaryotic Cell* and *Molecular and Cellular Biology*.

Transposable elements, plasmids, and restriction enzymes. Nomenclature of transposable elements (insertion sequences, transposons, and phage Mu, etc.) should follow the recommendations of Campbell et al. (Gene 5:197–206, 1979), with the modifications given in section vi of "Bacteria," above. The Internet site where insertion sequences of eubacteria and

archaea are described and new sequences can be recorded is http://www-is.biotoul.fr/is.html.

The system of designating transposon insertions at sites where there are no known loci, e.g., *zef-123*::Tn*5*, has been described by Chumley et al. (Genetics **91**:639–655, 1979). The nomenclature recommendations of Novick et al. (Bacteriol. Rev. **40**:168–189, 1976) for plasmids and plasmid-specified activities, of Low (Bacteriol. Rev. **36**:587–607, 1972) for F′ factors, and of Roberts et al. (Nucleic Acids Res. **31**:1805–1812, 2003) for restriction enzymes, DNA methyltransferases, homing endonucleases, and their genes should be used whenever possible. The nomenclature for recombinant DNA molecules, constructed *in vitro*, follows the nomenclature for insertions in general. DNA inserted into recombinant DNA molecules should be described by using the gene symbols and conventions for the organism from which the DNA was obtained.

Tetracycline resistance determinants. The nomenclature for tetracycline resistance determinants is based on the proposal of Levy et al. (Antimicrob. Agents Chemother. **43**:1523–1524, 1999). The style for such determinants is, e.g., Tet B; the space helps distinguish the determinant designation from that for phenotypes and proteins (TetB). The above-referenced article also gives the correct format for genes, proteins, and determinants in this family.

ABBREVIATIONS AND CONVENTIONS

Verb Tense

ASM strongly recommends that for clarity you use the **past** tense to narrate particular events in the past, including the procedures, observations, and data of the study that you are reporting. Use the present tense for your own general conclusions, the conclusions of previous researchers, and generally accepted facts. Thus, most of the abstract, Materials and Methods, and Results will be in the past tense, and most of the introduction and some of the Discussion will be in the present tense.

Be aware that it may be necessary to vary the tense in a single sentence. For example, it is correct to say "White (30) demonstrat*ed* that XYZ cells *grow* at pH 6.8," "Figure 2 shows that ABC cells fail*ed* to grow at room temperature," and "Air *was* removed from the chamber and the mice *died*, which *proves* that mice *require* air." In reporting statistics and calculations, it is correct to say "The values for the ABC cells *are* statistically significant, indicating that the drug inhibit*ed* . . . ."

For an in-depth discussion of tense in scientific writing, see *How To Write and Publish a Scientific Paper*, 7th ed.

Abbreviations

General. Abbreviations should be used as an aid to the reader, rather than as a convenience to the author, and therefore their **use should be limited.** Abbreviations other than those recommended by the IUPAC-IUB (*Biochemical Nomenclature and Related Documents*, 1992) should be used only when a case can be made for necessity, such as in tables and figures.

It is often possible to use pronouns or to paraphrase a long word after its first use (e.g., "the drug" or "the substrate"). Standard chemical symbols and trivial names or their symbols (folate, Ala, and Leu, etc.) may also be used.

Define each abbreviation and introduce it in parentheses the first time it is used; e.g., "cultures were grown in Eagle minimal essential medium (MEM)." Generally, eliminate abbreviations that are not used at least three times in the text (including tables and figure legends).

Not requiring introduction. In addition to abbreviations for Système International d'Unités (SI) units of measurement, other common units (e.g., bp, kb, and Da), and chemical symbols for the elements, the following should be used without definition in the title, abstract, text, figure legends, and tables: DNA (deoxyribonucleic acid); cDNA (complementary DNA); RNA (ribonucleic acid); cRNA (complementary RNA); RNase (ribonuclease); DNase (deoxyribonuclease); rRNA (ribosomal RNA); mRNA (messenger RNA); tRNA (transfer RNA); AMP, ADP, ATP, dAMP, ddATP, and GTP, etc. (for the respective 5′ phosphates of adenosine and other nucleosides) (add 2′-, 3′-, or 5′- when needed for contrast); ATPase and dGTPase, etc. (adenosine triphosphatase and deoxyguanosine triphosphatase, etc.); NAD (nicotinamide adenine dinucleotide); NAD^+ (nicotinamide adenine dinucleotide, oxidized); NADH (nicotinamide adenine dinucleotide, reduced); NADP (nicotinamide adenine dinucleotide phosphate); NADPH (nicotinamide adenine dinucleotide phosphate, reduced); $NADP^+$ (nicotinamide adenine dinucleotide phosphate, oxidized); poly(A) and poly(dT), etc. (polyadenylic acid and polydeoxythymidylic acid, etc.); oligo(dT), etc. (oligodeoxythymidylic acid, etc.); UV (ultraviolet); PFU (plaque-forming units); CFU (colony-forming units); MIC (minimal inhibitory concentration); Tris [tris(hydroxymethyl)aminomethane]; DEAE (diethylaminoethyl); EDTA (ethylenediaminetetraacetic acid); EGTA [ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid]; HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid); PCR (polymerase chain reaction); and AIDS (acquired immunodeficiency syndrome). Abbreviations for cell lines (e.g., HeLa) also need not be defined.

The following abbreviations should be used without definition in tables:

amt (amount)
approx (approximately)
avg (average)
concn (concentration)
diam (diameter)
expt (experiment)
exptl (experimental)
ht (height)
mo (month)
mol wt (molecular weight)
no. (number)
prepn (preparation)
SD (standard deviation)
SE (standard error)
SEM (standard error of the mean)
sp act (specific activity)
sp gr (specific gravity)
temp (temperature)
tr (trace)
vol (volume)
vs (versus)
wk (week)
wt (weight)
yr (year)

Drugs and pharmaceutical agents. Should an author decide to abbreviate the names of antimicrobial agents in a manuscript, the following standard abbreviations are strongly recommended.

(i) Antibacterial agents. Amikacin, AMK; amoxicillin, AMX; amoxicillin-clavulanic acid, AMC; ampicillin, AMP; ampicillin-sulbactam, SAM; azithromycin, AZM; azlocillin, AZL; aztreonam, ATM; carbenicillin, CAR; cefaclor, CEC; cefadroxil, CFR; cefamandole, FAM; cefazolin, CFZ; cefdinir, CDR; cefditoren, CDN; cefepime, FEP; cefetamet, FET; cefixime, CFM; cefmetazole, CMZ; cefonicid, CID; cefoperazone, CFP; cefotaxime, CTX; cefotetan, CTT; cefoxitin, FOX; cefpodoxime, CPD; cefprozil, CPR; ceftazidime, CAZ; ceftibuten, CTB; ceftizoxime, ZOX; ceftriaxone, CRO; cefuroxime (axetil or sodium), CXM; cephalexin, LEX; cephalothin, CEF; cephapirin, HAP; cephradine, RAD; chloramphenicol, CHL; cinoxacin, CIN; ciprofloxacin, CIP; clarithromycin, CLR; clinafloxacin, CLX; clindamycin, CLI; colistin, CST; daptomycin, DAP; dicloxacillin, DCX; dirithromycin, DTM; doxycycline, DOX; enoxacin, ENX; erythromycin, ERY; fleroxacin, FLE; fosfomycin, FOF; gatifloxacin, GAT; gentamicin, GEN; grepafloxacin, GRX; imipenem, IPM; kanamycin, KAN; levofloxacin, LVX; linezolid, LZD; lomefloxacin, LOM; loracarbef, LOR; meropenem, MEM; methicillin, MET; mezlocillin, MEZ; minocycline, MIN; moxalactam, MOX; moxifloxacin, MXF; nafcillin, NAF; nalidixic acid, NAL; netilmicin, NET; nitrofurantoin, NIT; norfloxacin, NOR; ofloxacin, OFX; oxacillin, OXA; penicillin, PEN; piperacillin, PIP; piperacillin-tazobactam, TZP; polymyxin B, PMB; quinupristin-dalfopristin (Synercid), Q-D; rifabutin, RFB; rifampin, RIF; rifapentine, RFP; sparfloxacin, SPX; spectinomycin, SPT; streptomycin, STR; teicoplanin, TEC; telithromycin, TEL; tetracycline, TET; ticarcillin, TIC; ticarcillin-clavulanic acid, TIM; tigecycline, TGC; tobramycin, TOB; trimethoprim, TMP; trimethoprim-sulfamethoxazole, SXT; trovafloxacin, TVA; and vancomycin, VAN.

(ii) β-Lactamase inhibitors. Clavulanic acid, CLA; sulbactam, SUL; and tazobactam, TZB.

(iii) Antifungal agents. Amphotericin B, AMB; clotrimazole, CLT; flucytosine, 5FC; fluconazole, FLC; itraconazole, ITC; ketoconazole, KTC; nystatin, NYT; terbinafine, TRB; and voriconazole, VRC.

(iv) Antiviral agents. Acyclovir, ACV; cidofovir, CDV; famciclovir, FCV; foscarnet, FOS; ganciclovir, GCV; penciclovir, PCV; valacyclovir, VCV; and zidovudine, AZT.

The use of "nonstandard" abbreviations to designate names of antibiotics and other pharmaceutical agents generally will not be accepted, because the use of different abbreviations for a single agent has often caused confusion. If, on occasion, a nonstandardized abbreviation for a drug or pharmaceutical substance is used, it will be accepted under the following conditions: (i) it must be defined at the first use in the text, (ii) it must be unambiguous in meaning, and (iii) it must contribute to ease of assimilation by readers.

Chemical or generic names of drugs should be used; the use of trade names is not permitted. Avoid the ambiguous term "generation" when classes of drugs are described. When code names or corporate proprietary numbers are to be used, either the chemical structure of the compound or a published literature reference illustrating the chemical structure, if known, must be provided at the first occurrence of the code name or number. For compounds not identified by generic nomenclature, all previous or concurrent identification numbers or appellations should be listed in the manuscript.

Pharmacodynamic terminology. Pharmacodynamic indices (PDIs) must be introduced at their first occurrence in the text and follow guidelines set forth by Mouton et al. (J. Antimicrob. Chemother. 55:601–607, 2005). In Materials and Methods, it should be clearly stated how the PDIs were derived. The most common indices used are the following: AUC/MIC ratio (the area under the concentration-time curve over 24 h in steady state divided by the MIC), AUIC (the area under the inhibitory curve; note that the AUC/MIC ratio is not equal to the AUIC), $\%T_{MIC}$ (the cumulative percentage of a 24-h period that the drug concentration exceeds the MIC under steady-state pharmacokinetic conditions), C_{max}/MIC ratio (the peak level divided by the MIC), PTA (probability of target attainment), and CFR (cumulative fraction of response). Clear distinction should be made between $\%T_{MIC}$, which is expressed as a percentage of the dosing interval, and T_{MIC}, expressed in hours. It is strongly recommended that the prefix *f* be used with an index (e.g., *f*AUC) if the free, unbound fraction of the drug is meant.

β-Lactamases

Studies performed to characterize a β-lactamase or the interaction of a compound with a β-lactamase (i.e., as a substrate, inhibitor, or inducer) should follow the guidelines set forth by Bush and Sykes (Antimicrob. Agents Chemother. 30:6–10, 1986). Assays that measure the hydrolysis of β-lactam antibiotics must be appropriate for the substrate examined (e.g., iodometric methods are not appropriate quantitative assays for substrates whose products are unknown). Reproducibility of results must be shown. When referring to β-lactamases, please use the functional designations defined by Bush et al. (Antimicrob. Agents Chemother. 39:1211–1233, 1995). Alternatively, if the amino acid sequence for the enzyme is known, the β-lactamases may be described by molecular class as initiated by Ambler (Philos. Trans. R. Soc. Lond. B Biol. Sci. 289:321–331, 1980).

A database of defining amino acid alterations for many β-lactamases is maintained at the Internet address http://www.lahey.org/studies/. The managers of that site should be consulted about the name of a potentially novel β-lactamase sequence before a new designation or number is proposed for publication.

In Vitro Susceptibility Tests

Tabulate results of determinations of minimal inhibitory and bactericidal concentrations according to the range of concentrations of each antimicrobial agent required to inhibit or kill the members of a species or of each group of microorganisms tested, as well as the corresponding concentrations required to inhibit 50 and 90% of the strains (MIC_{50} and MIC_{90}, respectively) and those required to kill 50 and 90% of the strains (MBC_{50} and MBC_{90}, respectively). The MIC_{50} and MIC_{90} re-

ported should be the actual concentrations tested that inhibited 50 and 90%, respectively, of the strains. They should not be values calculated from the actual data obtained. When only six to nine isolates of a species are tested, tabulate only the MIC range of each antimicrobial agent tested.

If more than a single drug is studied, insert a column labeled "Test agent" between the columns listing the organisms and the columns containing the numerical data and record data for each agent in the same isolate order. Cumulative displays of MICs or MBCs in tables or figures are acceptable only under unusual circumstances.

The percentage of strains susceptible and/or resistant to an antibiotic at its breakpoint concentration may be given only if an appropriate breakpoint has been approved, as by the Clinical and Laboratory Standards (http://www.clsi.org/). In the absence of approved breakpoints, authors cannot assign breakpoints or use breakpoints from related antibiotics. An exploratory analysis tabulating the percentage of strains inhibited over a range of concentrations is acceptable.

Bactericidal tests must be performed with a sufficient inoculum (>5×10^5 CFU/ml) and subculture volume (0.01 ml) to ensure accurate determination of the 99.9% killing endpoint, as described by Pearson et al. (Antimicrob. Agents Chemother. 18:699–708, 1980) and Taylor et al. (Antimicrob. Agents Chemother. 23:142–150, 1983). Inoculum size and subculture volume are also critical to studies of combinations of antimicrobial agents.

Synergy is defined in two-dimensional or checkerboard tests when the fractional inhibitory concentration (FIC) or fractional bactericidal concentration (FBC) index (Σ) is ≤0.5. In killing curves, synergy is defined as a ≥2-$\log_{10}$ decrease in CFU per milliliter between the combination and its most active constituent after 24 h, and the number of surviving organisms in the presence of the combination must be ≥2 $\log_{10}$ CFU/ml below the starting inoculum. At least one of the drugs must be present in a concentration which does not affect the growth curve of the test organism when used alone. Antagonism is defined by a ΣFIC or ΣFBC of >4.0.

When standard twofold-dilution schemes are used to determine checkerboard interactions, the inherent variability of the method casts doubt on the significance of interactions represented by ΣFICs or ΣFBCs of >0.5 but ≤4. Therefore, such interactions, if labeled at all, should be termed "indifferent." Alternatively, indices in this range may be described as "nonsynergistic" or "nonantagonistic," as appropriate. The technically imprecise term "additive" should be avoided, as it is too easily misunderstood. See reports by W. R. Greco et al. (Pharmacol. Rev. 47:331–385, 1995), F. C. Odds (J. Antimicrob. Chemother. 52:1, 2003), and M. D. Johnson et al. (Antimicrob. Agents Chemother. 48:693–715, 2004) for further discussion of these issues.

For killing curve tests, the minimal, accurately countable number of CFU per milliliter must be stated and the method used for determining this number must be described. In the absence of any drug and with a sample size of 1 ml, this number is 30 (1.5 in $\log_{10}$) CFU. If procedures for drug inactivation or removal have not been performed, the author must state how drug carryover effects were eliminated or quantified. For drugs showing an inoculum effect, mere dilution below the MIC obtained in standard tests is not sufficient.

Clinical Trials

(i) Registration. AAC requires the prospective registration (i.e., before the first patient is enrolled) of a clinical trial in a public trials registry in accordance with guidelines established by the International Committee of Medical Journal Editors (ICMJE) (http://www.icmje.org/publishing_10register.html). The ICMJE defines a clinical trial as "any research project that prospectively assigns human subjects to intervention or concurrent comparison or control groups to study the cause-and-effect relationship between a medical intervention and a health outcome." Such intervention may include drugs, surgical procedures, devices, behavioral treatments, process-of-care changes, etc.

AAC does not require registration in a particular registry, but the registry chosen must meet the following criteria, in agreement with ICMJE recommendations. It must be (a) accessible to the public free of charge, (b) open to all registrants, (c) managed by a not-for-profit organization, (d) monitored by a mechanism to ensure validity of registration data, and (e) searchable electronically. A registration with missing fields or uninformative terminology will be deemed inadequate.

The registry and the trial registration number must be included at the end of the abstract. If a registration number is available, the authors should state this number the first time a trial acronym is used to refer to the trial being reported or to other trials mentioned in the manuscript.

(ii) Criteria for enrollment. The methods used to find and enroll patients and the criteria for enrollment in a clinical trial should be stated. In addition, the time period (month/year to month/year) of the enrollment should be specified. It should be indicated, if appropriate, that written informed consent was obtained and that the trial was approved by the pertinent committee on human subjects.

(iii) Method of randomization. Randomized, double-blind studies are preferred. Comparisons using historical controls are usually regarded as questionable unless the differences in outcome between the groups are dramatic and almost certainly the result of the new intervention. The rationale for the choice of the control group should be explained. The sample size should be justified, and the method of randomization should be stated.

(iv) Criteria for determining whether a case is evaluable. The minimum criteria for evaluability should be stated explicitly. For example, it should be stated that the minimum criterion for evaluability was *a* or the combination of *b* and *c* rather than *a*, *b*, and *c* without designating which were the minimum criteria. The criteria for evaluability are usually different from those for enrollment.

(v) Reasons for nonevaluability. State the number of patients in each group who were excluded from evaluation and the reason(s) for each exclusion.

(vi) Criteria for assessment. Define each outcome for each category of assessment (e.g., "clinical outcomes were classified as cure, improvement, and failure; microbiological outcomes were classified as eradication, persistence, and relapse"). The frequency and timing of such assessments in relation to treatment should be stated. Specify any changes made in the study regimen(s) during the trial; the results for regimens with and without such modification generally should be stated separately. The criteria (questionnaires, results of specific laboratory tests) for evaluation of adverse effects should be stated, as should the period encompassed in the assessment and the time of assessment in relation to the time of treatment (e.g., daily during treatment). Some authors prefer to consider superinfections as failures of treatment, whereas others prefer to consider them separately or even as adverse effects. In any event, the manuscript should state the number of superinfections with each regimen and should differentiate between superinfections and colonization. The duration of follow-up should be mentioned.

(vii) Statistical analyses. The type of statistical test should be stated, and when appropriate, the reason for the choice of test should be given. References should be given for statistical procedures other than the *t* test, chi-square test, and Wilcoxon rank sum test. The comparability of the treatment groups at the baseline should be evaluated statistically.

For a review of some common errors associated with statistical analyses and reports, plus guidelines on how to avoid them, see the article by Olsen (Infect. Immun. 71:6689–6692, 2003).

For a review of basic statistical considerations for virology experiments, see the article by Richardson and Overbaugh (J. Virol. 79:669–676, 2005).

(viii) Beta error. For trials which show no statistically significant difference between regimens, the authors should calculate the probability (β) of a type II error and the power of the study ($1 - \beta$) to detect a specified clinically meaningful difference in efficacy between the regimens. For further details, see the article by Freiman et al. (N. Engl. J. Med. 299:690–694, 1978). Alternatively, or in addition, the authors should indicate the magnitude of difference between the regimens that could have been detected at a statistically significant level with the number of evaluable patients studied.

For further details, see the editorial on guidelines for clinical trials (Antimicrob. Agents Chemother. 33:1829–1830, 1989).

Reporting Numerical Data

Standard metric units are used for reporting length, weight, and volume. For these units and for molarity, use the prefixes m, μ, n, and p for 10^{-3}, 10^{-6}, 10^{-9}, and 10^{-12}, respectively. Likewise, use the prefix k for 10^{3}. Avoid compound prefixes such as mμ or μμ. Use μg/ml or μg/g in place of the ambiguous ppm. Units of temperature are presented as follows: 37°C or 324 K.

When fractions are used to express units such as enzymatic activities, it is preferable to use whole units, such as g or min, in the denominator instead of fractional or multiple units, such as μg or 10 min. For example, "pmol/min" is preferable to "nmol/10 min," and "μmol/g" is preferable to "nmol/μg." It is also preferable that an unambiguous form, such as exponential notation, be used; for example, "$\mu mol\ g^{-1}\ min^{-1}$" is preferable to "μmol/g/min." Always report numerical data in the appropriate SI units.

Representation of data as accurate to more than two significant figures must be justified by presentation of appropriate statistical analyses.

For a review of some common errors associated with statistical analyses and reports, plus guidelines on how to avoid them, see the article by Olsen (Infect. Immun. 71:6689–6692, 2003).

For a review of basic statistical considerations for virology experiments, see the article by Richardson and Overbaugh (J. Virol. 79:669–676, 2005).

Isotopically Labeled Compounds

For simple molecules, labeling is indicated in the chemical formula (e.g., $^{14}CO_2$, $^{3}H_2O$, and $H_2{}^{35}SO_4$). Brackets are not used when the isotopic symbol is attached to the name of a compound that in its natural state does not contain the element (e.g., ^{32}S-ATP) or to a word that is not a specific chemical name (e.g., ^{131}I-labeled protein, ^{14}C-amino acids, and ^{3}H-ligands).

For specific chemicals, the symbol for the isotope introduced is placed in square brackets directly preceding the part of the name that describes the labeled entity. Note that configuration symbols and modifiers precede the isotopic symbol. The following examples illustrate correct usage:

[^{14}C]urea	[γ-^{32}P]ATP
L-[*methyl*-^{14}C]methionine	UDP-[U-^{14}C]glucose
[2,3-^{3}H]serine	*E. coli* [^{32}P]DNA
[α-^{14}C]lysine	fructose 1,6-[1-^{32}P]bisphosphate

Title: An *in vitro* evaluation of the induction of dormant ring stages in *Plasmodium falciparum* parasites *in vitro* by artemisone and artemisone entrapped in Pheroid®.

Running title: The induction of dormancy by artemisone.

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Abstract

Artemisinin and its derivatives are the most potent antimalarial drugs currently available but are associated with high rates of *in vivo* recrudescence following monotherapy. A plausible explanation for this recrudescence is the dormancy phenomenon, where the artemisinins temporarily arrest the development of ring stage parasites. The effect of the Pheroid on the *in vitro* antimalarial activities of artemisone and on the ability of artemisone to induce dormancy was evaluated. The *in vitro* antimalarial activities of artemisone and its active metabolite M1 were compared with that of artesunate and dihydroartemisinin against sensitive and multidrug resistant strains of *Plasmodium falciparum*. Artemisone and its active metabolite M1 were also evaluated for its ability to induce dormancy in the chloroquine-resistant W2 *P. falciparum* laboratory strain. The Pheroid did not influence the *in vitro* activity or induction of dormancy of artemisone. Artemisone abruptly arrested parasite growth and induced dormant ring stages in a similar manner to dihydroartemisinin. Nevertheless, artemisone was the most active of the three artemisinin derivatives evaluated.

1. Introduction

Artemisinin and its derivatives are the most potent and rapidly acting drugs for the treatment of *Plasmodium falciparum* malaria, the most prevalent and lethal of Plasmodium species[1]. The artemisinins reduce the parasitic load by up to 10,000-fold per asexual cycle and until recently most patients became blood smear negative within 3 days of commencing daily artesunate treatment[2]. However, this class of antimalarial drugs is associated with high recrudescence. To circumvent the high rate of recrudescence (up to 50% after artesunate monotherapy)[3], artemisinin-based combination therapies (ACTs) are now used worldwide for the first-line treatment of uncomplicated *P. falciparum* malaria[4].

ACTs are effective because the artemisinin component reduces the parasite load rapidly, while the longer half-life partner drug eliminates the remaining parasites[4]. However, there are serious concerns about the emergence of artemisinin resistance in Southeast Asia (Cambodia, Myanmar, Thailand and Viet Nam), as evidenced by reports

of prolonged *P. falciparum* parasite clearance time *in vivo*[4,5,6,7], which is currently the best marker of resistance. Genetic investigations have yet to uncover molecular markers for artemisinin resistance[8]. Testing patient isolates with and without prolonged parasite clearance times by standard *in vitro* assays have revealed inconsistent findings: no significant differences in DHA 50% inhibitory concentrations (IC_{50}) were reported[6], whereas there were several-fold differences in IC_{50} values reported later[9].

It is not clear why patients treated with artemisinins experience high rates of recrudescences, because retreatment with artemisinins is generally effective in eliminating parasites. A plausible explanation for recrudescence is a quiescent state or dormancy that protects ring stage parasites against artemisinin exposure[2,10,11]. The artemisinin-treated ring stages of *P. falciparum* have the ability to enter a temporary growth arrest or dormant state, wherein they can survive drug treatment. These parasites are capable of resuming normal growth once drug pressure is removed[10,11,12,13]. In support of the dormancy concept microarray studies show that these dormant rings enter into transcriptional arrest until parasite growth is resumed[14,15]. This dormancy phenomenon is linked specifically to artemisinins since other classes of antimalarial drugs such as quinine do not induce dormancy[12]. Dormant parasites with similar morphology to those formed *in vitro* are also observed *in vivo* in rodent malaria models treated with artesunate[16].

Artemisone, a 10-alkylaminoartemisinin, is a new artemisinin derivative that has a potent antiplasmodial activity, superior *in vivo* elimination half-life, good oral bioavailability and metabolic stability, and no neurotoxicity[17] (Figure 1). Artemisone is well-tolerated in humans[18] with a curative effective dose of approximately one-third that of artesunate[19]. Thus, artemisone appears to be an attractive candidate partner drug for fixed-dose ACTs.

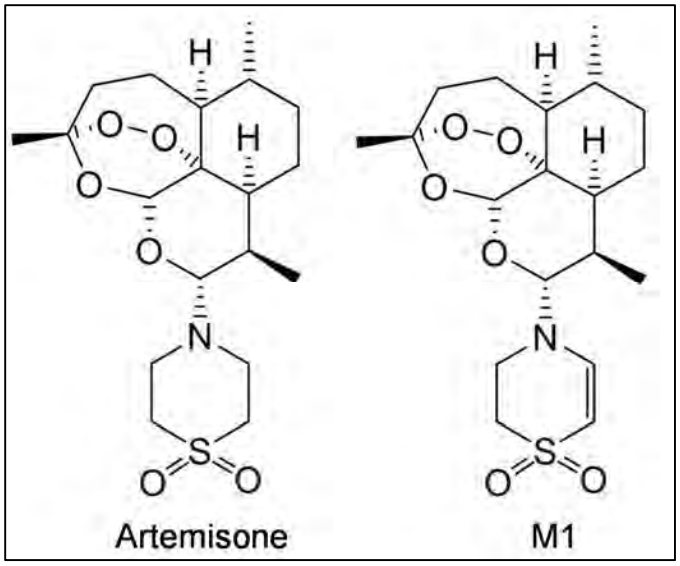

Figure 1: Structure diagrams of artemisone and its metabolite M1

The problem of parasite recrudescence after artemisinin monotherapy is a common trait amongst all artemisinins[10]. This also appears to be the same for artemisone based on *in vivo* studies in non-human primates. In a study where Aotus monkeys were treated with the chloroquine-resistant FVO strain of *P. falciparum* with either artesunate (n=3 monkeys) or artemisone (n=4 monkeys) at 10 mg/kg/day for 3 days, the artemisone-treated group cleared parasites within 24 h, whereas parasites were still present after 48 h in the artesunate-treated group. One monkey in the artemisone group was cured, and the other monkeys recrudesced between days 20 and 29. In contrast to artemisone, all monkeys recrudesced after artesunate treatment, with parasites reappearing between 9 and 20 days. When artemisone (10 mg/kg) was combined with mefloquine (5 mg/kg) as a single oral dose or was given 10 mg/kg/day with amodiaquine (20 mg/kg/day) for 3 days, the monkeys were completely cured[20].

It is well known that drug formulation can markedly affect the oral bioavailability and the efficacy of drugs. By using liposomes as the drug delivery system, both the pharmacokinetics and antimalarial efficacy of artemisinin was enhanced[21,22]. Self-emulsifying drug delivery systems also enhanced the antimalarial efficacy of β-arteether[23]. Liposomes containing β-artemether prevented malaria recrudescence in *Plasmodium chabaudi* infected mice[24].

Entrapment of anti-infective agents for the treatment of HIV[25], tuberculosis[25,26] and malaria[25,27,28,29,30,31,32,33,34,35] into Pheroid® technology has been reported to increase the efficacy and/or oral bioavailability of drugs. In particular, entrapment of chloroquine[27,28,32,33], amodiaquine[27,32], mefloquine[27,28,30], artemeter[27,29,30,33], artemiside[29], artesunate[27,29,31] and artemisone[29] showed significant enhancement of *in vitro* efficacy in *Plasmodium falciparum* strains. The entrapment of amodiaquine[32] and artemisone[34] showed significant enhancement of *in vivo* bioavailability in rodents and, in the case of chloroquine, in vervet monkeys[35].

Pheroid® technology is a lipid-based colloidal drug delivery system capable of self-assembly that is able to capture, transport and deliver active pharmaceutical ingredients. Pheroid® contains mainly vitamin F ethyl ester), polyethoxylated castor oil (Kollifor), α-tocopherol, nitrous oxide-saturated water and occasionally polyethylene glycol (PEG)[363738]. By varying the ratios of components and/or the preparation process, the size, structure and type of the Pheroid® formulations can be manipulated[26]. Pheroid® vesicles, microsponges or pro-Pheroid® can be prepared. The pro-Pheroid® formulations consist of an oil phase saturated with nitrous oxide gas and upon addition of the water phase, Pheroid® micro- and nano-particles form spontaneously[26].

In this study, we investigated the effect of Pheroid® on the *in vitro* antimalarial activity of artemisone and its effect on dormancy. If the Pheroid prevent the induction of dormancy *in vitro*, it may circumvent the high rate of recrudescence of artemisinins *in vivo*. We investigated the *in vitro* antimalarial activity of the active metabolite M1, and DHA against multidrug-resistant *P. falciparum* lines and examined whether artemisone or M1 induces formation of dormant ring stages *in vitro,* as has been reported for DHA.

2. Materials and methods

2.1. Drug preparation

The following drugs were used: chloroquine diphosphate (CQ) (Sigma-Aldrich, St. Louis, MO), mefloquine (MQ) (Sigma-Aldrich, St. Louis, MO), atovaquone (ATQ) (GlaxoSmithKline, Middlesex, UK), dihydroartemisinin (DHA) and artesunate (AS) (DK. Pharma, Hanoi, Vietnam). Artemisone (AMS) and the artemisone metabolite M1 were prepared by Wing-Chi Chan and Ho-Ning Wong of the Department of Chemistry, Hong Kong University of Science and Technology. All drugs were dissolved in 50% methanol, except for DHA which was dissolved in 100% methanol and atovaquone, which was dissolved in 100% DMSO. The stock concentrations for all drugs were 1 mM except for chloroquine (5 mM) and atovaquone (32 mM). For drug susceptibility assays drug dilutions were freshly prepared using hypoxanthine-free complete medium and the final solvent concentration were <0.01%.

The pro-Pheroid® containing artemisone (30 mM; AMS-Phe) was prepared from artemisone (0.16 g), PEG 400 (4.90 g), vitamin F ethyl ester (66.30 g), Kolliphor® EL (27.62 g), butylated hydroxyanisole (BHA; 0.01 g), butylated hydroxytoluene (BHT; 0.01 g) and dl-α-tocopherol (1 g). Artemisone was added to PEG-400, heated to 70℃ in a water bath, followed by sonication for 15 min. Vitamin F ethyl ester, Kolliphor EL, BHA and BHT was then added and heated to 70℃, followed by sonication for 15 min. Dl-α-tocopherol was added and the mixture gassed with nitrous oxide under pressure (200 kPA) for four days. Similarly, the drug-free pro-Pheroid® were prepared from PEG-400, (4.90 g), vitamin F ethyl ester (66.41 g), kollifor EL (27.67 g), BHA (0.01 g), BHT (0.01 g) and dl-α-tocopherol (1 g).

The Pheroid® drug dilutions were freshly prepared using hypoxanthine-free complete medium or hypoxanthine-free complete medium with added drug-free pro-Pheroid® to ensure that the oil (pro-Pheroid®):water phase concentration of 0.004% (v/v) were kept constant. The artemisone and DHA stock solutions were added to 0.4% drug-free pro-Pheroid® and diluted to a final pro-Pheroid® concentration of 0.004%, and the appropriate drug concentration with hypoxanthine-free complete medium.

The Pro-Pheroid® was mixed with 0.1 N hydrochloric acid (1:100 v/v) and the particle size of the resulting Pheroid® vesicles were measured using a Hydro Malvern Mastersizer 2000MU (Malvern Instruments Ltd, Malvern, Worcestershire, UK). Particles were stained with Nile Red and their morphology characterized by confocal laser scanning electron microscopy (CLSM, Nikon D-eclipse C1 confocal laser scanning microscope) using the method as previously described[39]. Spherical particles were observed for both artemisone entrapped Pheroid® and drug-free Pheroid® formulations with sizes of 4.39 ± 0.52 and 14.95 ± 0.83 nm respectively.

2.2. *In vitro* cultivation of *P. falciparum*

Plasmodium falciparum strains W2 (Indochina), D6 (Sierra Leone), 7G8 (Brazil) and TM90-C2B, TM91-C235 and TM93-C1088 (all from Thailand) were maintained in culture by using standard techniques[40] in complete medium containing 10.4 g/L RPMI-1640-LPLF powder (Gibco BRL), 5.97 g/L HEPES buffer (MP Biomedicals, USA), 2.0 g/L D-glucose (BDH chemicals, Australia), 0.05 g/L hypoxanthine (Sigma, USA) and 40 mg/L gentamycin (Pfizer, Australia), sodium bicarbonate solution (0.21%), drug-free heat inactivated human plasma (10%), pooled from various blood types, obtained from the Australian Red Cross Blood Service (Brisbane) supplemented with 4% human red blood cells (O^+). Parasites were routinely synchronized at ring stage every other day and prior to the start of each experiment using 5% (wt/vol) D-sorbitol[41].

2.3. *In vitro* antimalarial activity assay

In vitro antimalarial activity of the compounds was evaluated by an isotopic ^{3}H-hypoxanthine growth inhibition assay as previously described[42]. Briefly, 2-fold serial dilutions were made in hypoxanthine-free complete medium, or pro-Pheroid® containing (0.004%) hypoxanthine-free complete medium for Pheroid® treatments, in 96-well plates. *P. falciparum* culture was added to wells, so the final volume in each well was 100µL with a haematocrit of 2% and 1% parasitaemia. Plates were incubated at 37°C in a 5% O_2/ 5% CO_2/ 90% N_2 gas mixture for 24 h, at which point 20 µL (0.2 µCi/well) of ^{3}H-hypoxanthine (Perkin Elmer, USA) was added to each well. The plates were incubated for an additional 24 h. The experiment was terminated by placing the plates

in a -20°C freezer. After thawing the plates, the lysed cells were harvested onto glass fibre filter mats (Perkin Elmer, USA) using a 96-well cell harvester (Harvester 96 ™; Tomtec, Oxon, UK) and left to dry. Uptake of ^{3}H-hypoxanthine was measured by a scintillation counter (Microbeta 2, Perkin Elmer) after the addition of MeltiLex™ solid scintillant (Perkin Elmer). Three independent experiments were performed for each compound, each in triplicate. The IC_{50} and IC_{90} values were determined by estimating the drug concentrations that inhibited parasite ^{3}H-hypoxanthine uptake by 50% and 90%, respectively, relative to drug-free control cultures by fitting the counts values to sigmoidal dose-response curves generated with PRISM V5.0 software (Graphpad Software Inc., USA). Statistical comparison of the data was done by analysis of variance (ANOVA) at a significance level of $p < 0.05$, using PRISM V5.0 software (Graphpad Software Inc., USA).

2.4. *In vitro* drug dormancy

Cultures (10 mL) of ring stages (>95%) W2 parasites with initial parasitaemia of 1% and 4% haematocrit were exposed to DHA (700 nM, ~100 x IC_{90}; Tuescher *et al.*, 2010), artemisone (200, 400 and 800 nM), artemisone (200, 400 and 800 nM) entrapped into the Pheroid®, metabolite M1 (200, 400 and 800 nM) and mefloquine (4230 nM; ~100 x IC_{90}) for 6 h. Drug-free cultures were run in parallel as negative controls. After 6 h incubation, the drugs were removed by washing the cultures thrice with culture medium after which the red blood cell pellets were re-suspended in 10 mL of culture medium. Each culture (10 mL) was split into two equal aliquots.

To ensure that the parasites, which did not become dormant and continued to grow after treatment were removed, one set of cultures was exposed to 5% D-sorbitol. This was done when the parasites in the control culture had reached the late trophozoite-schizont stage (~33-36 h). After exposure to sorbitol for 5 min, the cultures were washed thrice with culture medium, resuspended in medium and returned to 37°C incubator.

Thin and thick blood films were prepared daily for 30 days from each culture, stained with Giemsa stain and examined under light microscopy to determine the parasitaemia until parasites had recovered to at least 1% parasitaemia. Two independent experiments were performed. Culture medium was replaced and red blood cells were added to cultures every seven days.

3. Results

3.1. *In vitro* antimalarial drug activity

The *in vitro* activity of artemisone, M1, artemisone and DHA entrapped into the Pheroid® as well as several reference antimalarial drugs against *P. falciparum* lines are summarized in Table 1. Of the drugs tested, artemisone had the greatest blood schizontocidal activity across the six *P. falciparum* strains, with different antimalarial drug-susceptibility profiles and geographical origins. Artemisone (IC_{50} range: 0.71 to 1.29 nM) showed a significantly higher activity of approximately 2 fold *in vitro* compared with either artesunate (IC_{50} range: 1.44 to 3.02 nM) or DHA (IC_{50} range: 1.33 to 2.26 nM). When compared to its parent drug, M1 was 3.4 to 6.6-fold less active against the *P. falciparum* lines.

The entrapment of artemisone in Pheroid® during manufacturing, as well as the addition of the Pheroid® to stock solutions of artemisone and DHA during preparation of final stock solutions, did not improve the *in vitro* antimalarial activities of the drugs (Table 1, Figure 2).

Table 1. *In vitro* antimalarial activity of the artemisone and standard drugs against six *P. falciparum* strains.

	D6		W2		7G8		TM93-C1088		TM91-C235		TM90-C2B	
	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}
AMS	1.0 ± 0.4	2.2 ± 0.5	1.3 ± 0.5	1.8 ± 0.6	0.8 ± 0.1	1.7 ± 0.2	0.7 ± 0.2	1.3 ± 0.3	1.1 ± 0.5	2.2 ± 0.9	1.1 ± 0.4	2.2 ± 0.9
AMS + Phe	1.1 ± 0.6	1.9 ± 0.9	1.5 ± 0.2	2.8± 1.3	n.d.	n.d.	n.d.	n.d.	1.2 ± 0.6	2.1 ± 0.9	1.1 ± 0.5	1.8 ± 0.8
AMS (Phe)	0.9 ± 0.7	1.9 ± 1.2	1.3 ± 1.0	1.8 ± 1.4	1.0 ± 0.4	1.8 ± 0.1	0.8 ± 0.1	1.6 ± 0.2	1.4 ± 0.7	2.4 ± 1.1	1.1 ± 0.6	2.0 ± 0.9
M1	4.7 ± 0.2	10.4 ± 2.4	6.6 ± 0.4	9.1 ± 1.9	2.6 ± 0.8	5.8 ± 1.8	2.5 ± 0.4	4.5 ± 0.8	5.0 ± 0.6	9.0 ± 4.0	8.6 ± 8.2	35 ± 49
DHA	1.7 ± 0.4	4.6 ± 1.8	2.2 ± 0.8	3.8 ± 0.8	1.3 ± 0.2	4.2 ± 1.7	1.4 ± 0.4	3.4 ± 0.6	2.3 ± 0.7	5.0 ± 2.0	2.0 ± 0.7	4.5 ± 1.7
DHA + Phe	1.7 ± 1.1	3.6 ± 2.1	2.0 ± 0.4	3.8 ± 0.8	n.d.	n.d.	n.d.	n.d.	2.3 ± 0.9	5.3 ± 1.5	2.0 ± 1.0	3.8 ± 2.0
CQ	16 ± 2	19 ± 5	195 ± 70	305 ± 95	84.1 ± 18.4	159 ± 26	360 ± 38	558 ± 69	70 ± 12	157 ± 30	95.1 ± 22.8	171 ± 45
MQ	n.d.	n.d.	n.d.	n.d.	5.4 ± 1.7	33 ± 16	16 ± 5	49 ± 13	107 ± 41	312 ± 126	130 ± 51	319 ± 130
ATQ	n.d.	n.d.	n.d.	n.d.	3.1 ± 0.9	17 ± 4	18830 ± 5102	53257 ± 8816	2.2 ± 0.7	8.8 ± 3.9	31850 ± 6833	81065 ± 32155
AS	n.d.	n.d.	3.0 ± 1.6	4.7 ± 2.2	1.5 ± 0.2	3.0 ± 0.5	1.4 ± 0.1	2.4 ± 0.4	2.9 ± 1.4	4.4 ± 1.7	2.5 ± 0.5	4.1 ± 1.0

* n.d., not determined; W2 is chloroquine-resistant, D6 is chloroquine-sensitive, 7G8 is chloroquine-resistant, TM90-C2B is atovaquone and chloroquine-resistant, TM91-C235 is chloroquine and mefloquine-resistant and TM93-C1088 is atovaquone and chloroquine-resistant. CQ, chloroquine; MQ, mefloquine; ATQ, atovaquone; AS, artesunate; DHA, dihydroartemisinin; M1, metabolite of artemisone; AMS, artemisone, Phe, Pheroid®. Values represent the mean IC_{50} ± SD (nM) and IC_{90} ± SD (nM) from three independent experiments performed in triplicate.

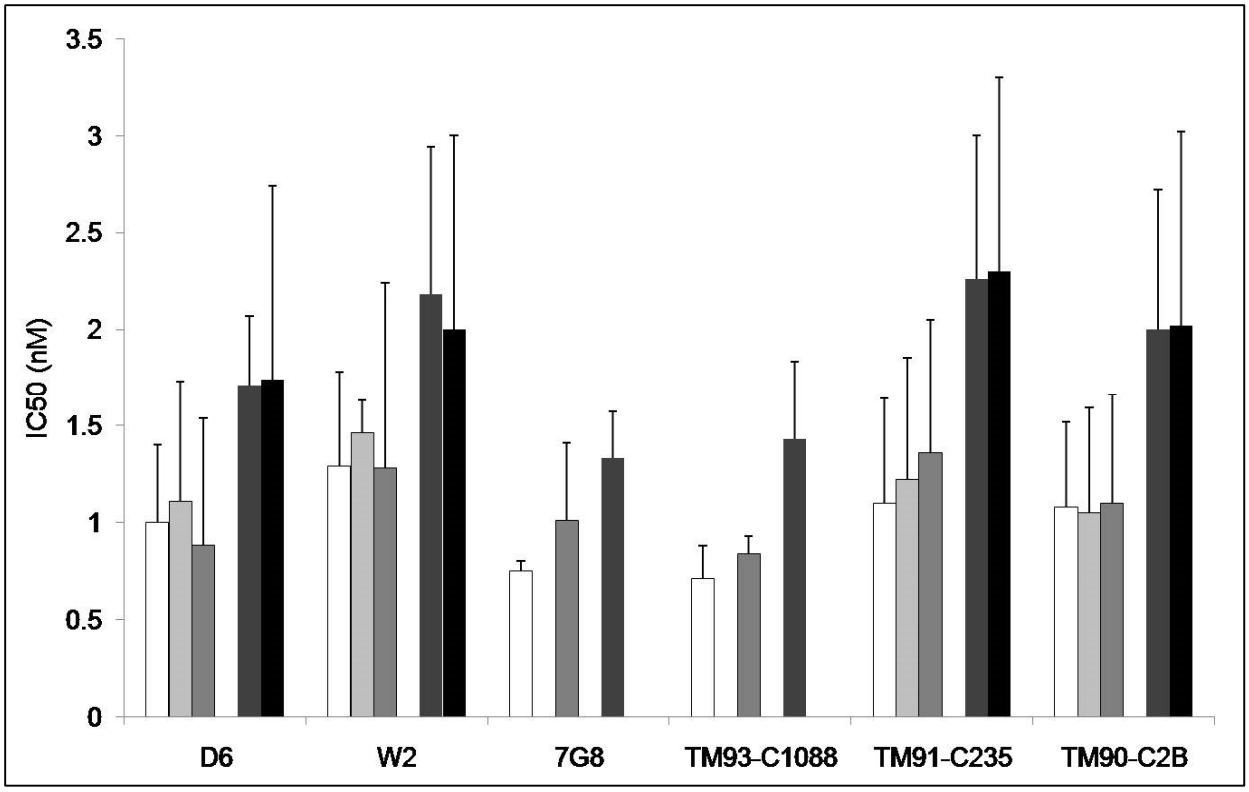

Figure 2 *In vitro* antimalarial activities of artemisone (white bars), artemisone with Pheroid® added separately (light grey bars), artemisone entrapped into the Pheroid® during preparation (medium grey bars), DHA (dark grey bars) and DHA with Pheroid® added separately (black bars) against six *P. falciparum* strains. The mean IC_{50} values from three independent experiments are shown. For the 7G8 and TM93-C1088 strains only artemisone, artemisone entrapped into the Pheroid® during manufacturing and DHA were assayed.

3.2. *In vitro* drug induced dormancy

The control parasites progressed from rings (start of experiment) to trophozoites at 24h, schizonts at ~33-36 h and after schizogony to rings again at 40 h after commencing the experiment. Following a single 6 h exposure to either DHA (700 nM), artemisone (200, 400 and 800 nM), artemisone entrapped into the Pheroid® (200, 400 and 800 nM) or metabolite M1 (200, 400 and 800 nM), parasite growth was arrested at ring stage, with morphologically abnormal rings and no trophozoites observed within at least 24 h after drug exposure (Figure 3). The majority of rings looked drug-affected with distinctly smaller nuclei and condensed rounded cytoplasm. Dormant rings, as previously described[10,15], were also seen in cultures treated with DHA, artemisone, artemisone entrapped into the Pheroid® and M1. Morpholgically, the dormant parasites had blue-stained cytoplasm with red nuclei. Unlike artemisinin derivatives, exposure of rings to mefloquine (4230 nM) did not result in immediate growth arrest, but stopped parasite development at the late ring early trophozoites stage (Figure 3).

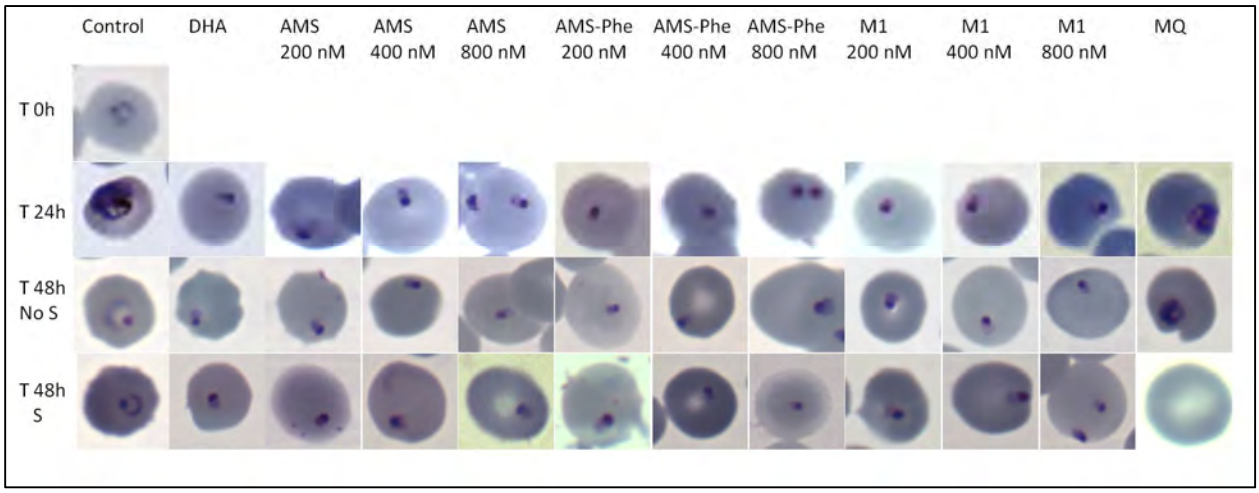

Figure 3 Comparison of control parasites with parasites exposed to DHA (dihydroartemisinin), artemisone (AMS), artemisone entrapped into the Pheroid® (AMS-Phe), artemisone metabolite M1, and MQ (mefloquine). No S, cultures not treated with sorbitol; S, cultures treated with sorbitol on day 2.

With the exception of mefloquine, growing parasites were first detected on thick blood films on day 3 after DHA, artemisone (200, 400 and 800 nM), artemisone entrapped into

the Pheroid® (200, 400 and 800 nM) and metabolite M1 (200, 400 and 800 nM) treatment and reached an initial parasitaemia (>1 %) by day 6 (Figure 4) without sorbitol treatment. With sorbitol-treatment that selectively kills all late-parasite stages except for ring forms, growing parasites were only detected on day 5 ± 1.4 and the time to reach initial parasitaemia of 1% was 9 ± 1.4 days. This delay in recovery indicates that there is a small number of parasites that are unaffected by the artemisinin compounds investigated. No parasites were observed on the thick blood smears of the mefloquine treated positive controls during the 30 day follow-up period.

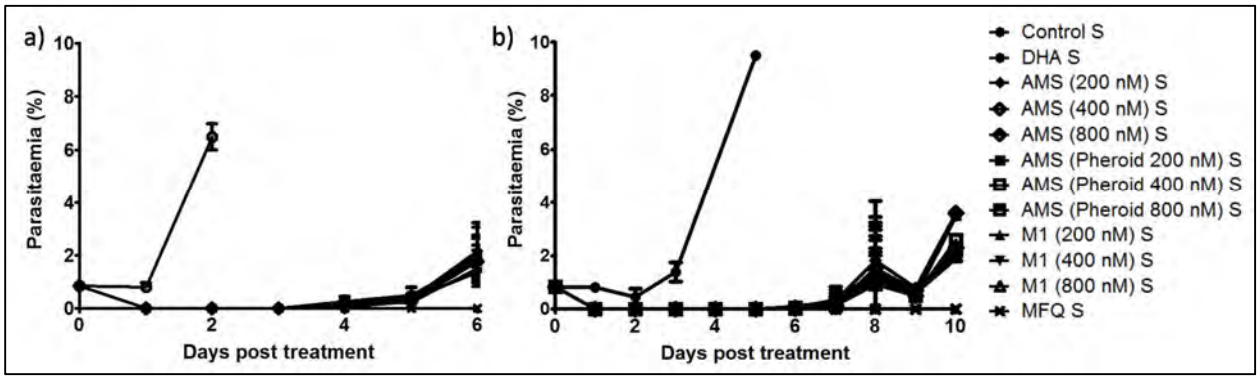

Figure 4 The effect of DHA (dihydroartemisinin), artemisone (AMS), artemisone entrapped into the Pheroid, metabolite M1 and MQ (mefloquine) against the *P. falciparum* W2 strain without (a) and with (b) sorbitol treatment on day 2.

4. Discussion

Our *in vitro* results are consistent with artemisone being the most potent artemisinin derivative available today[43,44]. In this study, we showed that artemisone was approximately 2-fold more potent than either artesunate or DHA. This compares well with the 2.4-fold greater antimalarial activity reported[45], but the fold difference was markedly less than that determined by Vivas and colleagues[43]. The IC_{50} values for chloroquine, mefloquine, and atovaquone were in good agreement with previous results against various *P. falciparum* lines[45,46,47,48,49,50,51,52]. The artemisone metabolite M1 was also highly active with IC_{50} values ranging from 2.5 to 8.6 nM, which is comparable to 4.2 ± 1.3 nM against the K1 strain of *P. falciparum*[18]. Previous *in vitro* findings obtained with an artemisone Pheroid® formulation were contradictory[29,33]. Steyn[29] reported a 4.5-

fold increase in the antimalarial activity of artemisone when entrapped into the Pheroid® vesicles, while Jourdan[33] reported equal activity. Therefore the in vivo results obtained during this study compares well with that of Jourdan[33].

Kyle and Webster[12] first reported that parasites survive in a dormant form for 3 to 8 days before resuming growth after treatment with artesunate or DHA, *in vitro*. This observation has led to the "dormancy" hypothesis, namely that parasites are able to survive artemisinin treatment by entering a state of dormancy, where they are protected from the drug's lethal effects and recover at a later stage to resume normal growth[53]. After a single 6 h exposure of ring stage parasites to 200 ng/mL (700 nM) DHA *in vitro*, a dose comparable to clinical DHA plasma concentrations after artesunate treatment, caused some parasites to enter a dormant state[10]. A small number of the dormant parasites recovered to become normal growing parasites within 3 to 20 days post-treatment. Teuscher and colleagues[10] also showed that after treating ring stage parasites with DHA for 6 h per day for 3 days, there was a 10-fold reduction in the recovery rate.

Further support of the artemisinin induced dormancy hypothesis for explaining the high level of recrudescence reported following monotherapy has been obtained using an in-host stochastic simulation model with the assumption that the *in vitro* dormancy rates and duration are applicable *in vivo*. Codd[11] and colleagues were was able to demonstrate that, following a single treatment with artemisinin, the proportion of parasitological and clinical failures were 77% and 67%, respectively. These theoretical failure rates rapidly decline with repeated treatment with the proportion of parasitological and clinical failures decreasing to 25% and 38%, respectively, after three days of artemisinin treatment. The predicted parasitological and clinical treatment failure rates in the simulated populations following three days of DHA treatment agreed with rates reported from several field trials[54,55]. These findings suggest that the *in vitro* dormancy rates following three short exposures to DHA appear to be a good predictor of the *in vivo* dormancy rate.

In the present study, we assessed the potential of artemisone to induce ring-stage dormancy in *P. falciparum*, *in vitro*. For the *in vitro* study, clinical relevant concentrations of artemisone and M1 were used, based on human data of a maximum plasma concentration of 140.2 ng/mL (~349.2 nM) for artemisone and 112.6 ng/mL (~281.9nM) for M1 after a three course of daily 80 mg artemisone to healthy subjects[18] . The *in vitro* dormancy data suggests that as in the case with artesunate and DHA, artemisone and its metabolite M1 also induce the formation of dormant ring stage parasites that are capable of surviving further drug treatment. Once drug pressure is removed, the ring-stage parasites resume development, and thereby initiate recrudescence. The Pheroid® delivery system containing artemisone performed in a similar fashion to that of artemisone in the induction of dormant ring-stage parasites and recovery. Artemisone does not seem to induce dormancy in a concentration dependent manner since all of the concentrations used reached initial parasitaemia within the same time period. Therefore, even though the Pheroid® enhances artemisone blood concentrations *in vivo*[34], it is likely that recrudescence will not be prevented.

Concerns of the high treatment failure rate (approximately 20%) of artesunate-mefloquine[56] and DHA-piperaquine[57] in western Cambodia is of great concern and highlights the urgent need to select better and more effective ACTs until potent replacement drugs can be developed. Although artemisone induces dormant ring stage parasites similar to DHA, the drug should be used in combination with a long acting partner drug to provide an alternative ACT. Artemisone is well-tolerated, has favourable pharmacokinetic properties, and is the most potent artemisinin available today[17,43,47,45]. The partner drug to be selected with artemisone should possess strong *in vitro* potency, have a relatively long half-life to prevent the induction and/or recovery of dormant ring-stages and be used as a fixed-dose treatment.

In summary, artemisone appears to be the most active artemisinin derivative against *P. falciparum in vitro*. However, similar to other artemisinins it induces dormant ring-stage parasites and if used alone recrudescences can be expected to occur. Artemisone administered in the Pheroid® delivery system does not prevent the induction of dormant

parasites and is no more active *in vitro* than the standard formulation of artemisone. The future of artemisone lies in the selection of a suitable partner drug that can prevent the induction and/or recovery of dormant parasites induced by the artemisinin.

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