Anticancer Properties of Distinct Antimalarial Drug Classes

Rob Hooft van Huijsduijnen1, R. Kiplin Guy2, Kelly Chibale3, Richard K. Haynes4, Ingmar Peitz5, Gerhard Kelter5, Margaret A. Phillips6, Jonathan L. Vennerstrom7, Yongyuth Yuthavong8, Timothy N. C. Wells1*

1 Medicines for Malaria Venture (MMV), Geneva, Switzerland, 2 St. Jude Children’s Research Hospital, Memphis, Tennessee, United States of America, 3 Department of Chemistry and Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Rondebosch, South Africa, 4 Centre of Excellence for Pharmaceutical Sciences, North-West University, Potchefstroom, South Africa, 5 Oncostex GmbH, Freiburg, Germany, 6 Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, Texas, United States of America, 7 Department of Pharmaceutical Sciences, Nebraska Medical Center, Omaha, Nebraska, United States of America, 8 BIOTEC, National Science and Technology Development Agency, Thailand Science Park, Pathumthani, Thailand

Abstract

We have tested five distinct classes of established and experimental antimalarial drugs for their anticancer potential, using a panel of 91 human cancer lines. Three classes of drugs: artemisinins, synthetic peroxides and DHFR (dihydrofolate reductase) inhibitors effected potent inhibition of proliferation with IC50s in the nM- low μM range, whereas a DHODH (dihydroorotate dehydrogenase) and a putative kinase inhibitor displayed no activity. Furthermore, significant synergies were identified with erlotinib, imatinib, cisplatin, dasatinib and vincristine. Cluster analysis of the antimalarials based on their differential inhibition of the various cancer lines clearly segregated the synthetic peroxides OZ277 and OZ439 from the artemisinin cluster that included artesunate, dihydroartemisinin and artemisone, and from the DHFR inhibitors pyrimethamine and P218 (a parasite DHFR inhibitor), emphasizing their shared mode of action. In order to further understand the basis of the selectivity of these compounds against different cancers, microarray-based gene expression data for 85 of the used cell lines were generated. For each compound, distinct sets of genes were identified whose expression significantly correlated with compound sensitivity. Several of the antimalarials tested in this study have well-established and excellent safety profiles with a plasma exposure, when conservatively used in malaria, that is well above the IC50s that we identified in this study. Given their unique mode of action and potential for unique synergies with established anticancer drugs, our results provide a strong basis to further explore the potential application of these compounds in cancer in pre-clinical or and clinical settings.

Citation: Hooft van Huijsduijnen R, Guy RK, Chibale K, Haynes RK, Peitz I, et al. (2013) Anticancer Properties of Distinct Antimalarial Drug Classes. PLoS ONE 8(12): e82962. doi:10.1371/journal.pone.0082962

Editor: Henk D. F. H. Schallig, Royal Tropical Institute, The Netherlands

Received August 19, 2013; Accepted October 22, 2013; Published December 31, 2013

Copyright: © 2013 Hooft van Huijsduijnen et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was partially supported by National Institutes of Health grants (U01AI075594; to MAP). No additional external funding was received for this study.

Competing Interests: The authors have the following interests. Ingmar Peitz and Gerhard Kelter are employed by Oncostex GmbH. Medicines for Malaria Venture is involved in supporting the development of some of these medicines. There are no patents or marketed products to declare. This does not alter the authors’ adherence to all the PLOS ONE policies on sharing data and materials.

* E-mail: wellst@mmv.org

Introduction

Over the past two decades, numerous studies have identified antitumor activities of malaria drugs. Nearly all these studies focused on artemisinin derivateives, which are based on natural sesquiterpene lactones with a 1,2,4-trioxane ring system. Originally isolated from Artemisia plants, this scaffold currently represents a cornerstone of the fight against malaria [1]. Artemisinin itself and its derivatives artsunate (ART), arteether, artemether and dihydroartemisinin (DHA) are, variously formulated, used in malaria. In addition, intense activity is aimed at exploring additional, synthetic peroxides. A considerable motivation for the interest in artemisinins in additional indications is their excellent, well-established safety profile.

The vast majority of studies with artemisinins for use in cancer (188 to date) involve in vitro and in vivo experiments aimed at establishing the drug’s mode of action and potential for synergy with established cancer drugs ([2–12]; see also the recent review [13]). By contrast, only a few clinical studies -mostly anecdotal findings from single cases, and one formal trial- have been performed ([14–20]; see [21] for a recent review of clinical uses), reporting modest improvement in patients with advanced non-small lung cancer. In light of the vast preclinical literature on anticancer properties of artemisinins and their excellent, well-established safety profile it is surprising that there are not more reports, or more widespread off-label use of artemisinins for cancer. As was pointed out recently [21] one issue with artemisinins is their short half-life in patients and variability in drug exposure between patients (eg [22,23]) and over time [24,25]. These problems are no major obstacle for eliminating Plasmodium parasites in malaria patients over a three-day cure, but may prevent efficient inhibition of metastasis-associated angiogenesis, if that were the principal mode of action for artemisinins’ use in cancer. The major issue had been the lack of registration for any indication in the US and the general lack of clinical grade material...
(produced under GMP conditions). There has been a recent step forward here with the WHO prequalification of artesunate for injection produced in China by Guilin in 2010, confirming that production is at internationally recognized GMP standards, and this product is available in Europe under special conditions. Sigma-Tau, as part of their collaboration with the US Army, has obtained an orphan drug designation for artesunate use against malaria from the United States FDA, and with an FDA filing planned for the first quarter in 2014 (Pietro Grossi, pers. comm.). This should pave the way to the registration of an artesunate for injection in the USA early in 2014.

In spite of extensive efforts and significant progress, the mode of action of artemisinins in eliminating intra-erythrocytic Plasmodium parasites, and their activity in cancer is still incompletely understood. For use in malaria, structure-activity relationships among analogs implicate the 1,2,4-trioxane peroxide pharmacophore as critical for artemisinins’ function [26]. One potential mechanism comes from the demonstration that interaction with free Fe$^{2+}$ or heme [27,28] triggers a chemical cascade that generates multiple, toxic reactive oxygen species (ROS; reviewed in [29]). Alternatively, or in parallel, artemisinins bind to heme and interfere with Plasmodium's critical detoxification of heme whereby toxic haptain is polymerized into an insoluble crystalline form of β-hematin called hemozin, also known as malaria pigment. Three anticaner mechanisms have been identified for artemisinins: A few reports suggest that artemisinins may steer the immune system from a Th2 to a Th1-dominated anticaner response with suppression of the Treg population [8,30]. Secondly, many studies find that artemisinins can interfere with angiogenesis [31–37] by repression of VEGF and Ang-1 secretion and interfering with choioallantoic membrane neovascularization. However, the vast majority of publications demonstrate that artemisinins specifically induce apoptosis in a variety of tumor cell lines (see [13] for a review). Molecular analysis indicates that artemisinins activate caspases 9 and 3 (but not caspase 8, which mediates the extrinsic/TNFR-related pathway). Again, activation with Fe$^{2+}$ appears to play a major role: preloading cancer cells with iron or iron-saturated holo-transferrin significantly potentiates the induction of apoptosis (eg [40–44]). The enhanced metabolic need for iron in cancer cells is well-established, and the up-regulated transferrin receptor is being investigated as a means to specifically target cancer cells [45,46]. Artemisinins appear to be directly activated by heme [47], as illustrated by the finding that compounds that bind mitochondrial heme also show good cytotoxicity [48]. The critical role of mitochondrial function in mediating the cytotoxicity of artemisinin was elegantly demonstrated in a yeast system [49]. Importantly, this mode of action is distinct from existing anticaner drugs. Consequently, artemisinins have a great potential to synergize with established treatments both in vitro and in vivo (eg [40,50–52]), even more importantly, multi-drug resistant cancers retain sensitivity towards artemisinins [53–56].

In order to explore the potential use of antimalarials in cancer it is important to further link their mode of action with the critical signaling pathways that drive cancer cell proliferation. This enables us to move beyond a simple and antiquated categorization of cell lines predominantly by the organ from which they originated. Linked to this, it is important to explore what the synergies with current anti-cancer drugs will be. The primary focus of this study was to test the semisynthetic artemisinins artesunate and its active metabolite dihydroartemisinin, the semisynthetic N-thiomorpholinio derivative, artemisone (BAY 44-9585 [57]) and two synthetic peroxides, OZ277 (also called arteholone) and OZA139. As a positive control, we also tested two anti-parasite dihydrofolate reductase inhibitors, pyrimethamine (recently shown to induce apoptosis in melanoma cells [58]) and P-218 [59], which were anticipated to show some activity against a human cancer cell panel. In addition we tested the highly selective parasite dihydroorotate dehydrogenase (DHODH) inhibitor DSM265 [60], and MMV 390048, a highly active anti-plasmodial compound which is a putative kinase inhibitor. Due to their high selectivity for parasite cells, these compounds were expected to be largely inactive. The cancer line panel comprised conventional commercially available cell lines as well as lines derived directly from patient-derived tumor xenografts (PDX). In order to gain insight in these molecules’ mode of action we correlated their potency with the cancer lines’ gene expression patterns. Finally we discuss our findings in the light of available pharmacokinetic data for these drug candidates and their clinical potential.

Materials and Methods

Tumor cell lines

The cell line panel comprised 68 cell lines derived from solid tumours and 24 cell lines derived from haematological malignancies [61]. Among the solid tumor cell lines, 30 were established at Oncotec from patient-derived tumor xenografts as described previously [61–63]. The other cell lines were either kindly provided by the NCI (Bethesda; MD) [64], or were purchased from ATCC (Rockville, MD), DSMZ (Braunschweig, Germany), ECACC (Salisbury, United Kingdom), KCLB (Seoul, Korea) or JCRB (Osaka, Japan). Authenticity of cell lines was verified at the DSMZ by STR (short tandem repeat) analysis, a PCR based DNA-fingerprinting methodology [65,66]. Cell lines were routinely passaged once or twice weekly and maintained in culture for up to 20 passages. All cells were grown at 37°C in a humidified atmosphere with 5% CO$_2$ in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum and 0.1 mg/mL gentamicin (medium and all components from PAA, Colbe, Germany).

Cell proliferation Assays

Cells were harvested from exponential phase cultures, counted and plated in 96 well flat-bottom microtiter plates at a cell density of 8,000–60,000 cells/well. After a 24 h recovery period to allow the cells to resume exponential growth, 10 μl of culture medium (six control wells/plate) or of culture medium with test compound(s) were added. The compounds were applied in duplicates at ten concentrations and treatment continued for four days. Determination of a potential synergism or antagonism by the median-effect principle of Chou-Talalay requires application of the combined substances at a constant, equipotent ratio, mostly at their IC$_{50}$ values. Mixtures at this constant ratio were tested at ten different dilutions in duplicate. Additionally, each compound was also tested in monotherapy on the same plate. Both compounds were added simultaneously to the cells. Of each test combination, two to three independent experiments were performed.

For solid tumor derived cell lines, a modified propidium iodide (PI) assay [67] was used to assess the anti-cancer activity of the compounds. After the 4 days treatment, cells were next washed with 200 μl PBS to remove dead cells, then 200 μl of a solution containing 7 μg/ml propidium iodide (PI) and 0.1% (v/v) Triton X-100 was added. After an incubation period of 1–2 hours at room temperature, fluorescence (F) was measured using the Cytofluor® 4000 microplate reader (excitation λ = 530 nm,
emission $\lambda = 620$ nm) to quantify the amount of attached viable cells.

For hematological cancer cell lines growing in suspension, the CellTiter-Blue® assay (#G8081, Promega) was used according to manufacturer’s instructions. After treatment of cells, 10 μl/well CellTiter-Blue® reagent was added. Following an incubation period of up to four hours, fluorescence (FU) was measured by using the EnVision Xcite multilabel reader (excitation $\lambda = 531$ nm, emission $\lambda = 615$ nm).

For calculations, the mean value of duplicate/quadruplicate (untreated control) data was used. Calculation of IC$_{50}$ values was done by 4 parameter non-linear curve fit (Oncotest Warehouse Software). The compounds were tested in 2–4 independent experiments and IC$_{50}$ values are shown as the mean of those experiments.

Assessment of drug-drug interaction (determination of CI according to Chou-Talalay)

Various two-drug combinations were evaluated using the median-effect principle proposed by Chou and Talalay [68]. This mathematical model, previously established for enzyme-substrate interactions, has been extended to multiple drug combinations. The formula of the median-effect principle is $f_a/f_u = [D/D_m]^m$, in which $f_a$ is the fraction of the cells affected, $f_u (1-f_a)$ is the fraction unaffected by the treatment, $D$ the drug concentration, $D_m$ the concentration required for 50% cell growth inhibition and $m$ the slope of the median-effect curve ($\log[f_a/f_u] = m \log D - m \log (D_m)$. The x-intercept yields $\log (D_m)$, and thus, the $D_m$ value. The deviation of data from the fitted median-effect equation is represented by the linear correlation coefficient $r$ of the median-effect plot. Usually the experimental data from cell culture experiments have $r > 0.90$.

The combination index (CI) was calculated by the Chou-Talalay equation, which takes into account both potency ($D_m$ or IC$_{50}$) and the shape of the dose-effect curve. The general equation for the classic isobologram (CI = 1) is given by: $CI = (D_1)_{IC_{50}} + (D_2)_{IC_{50}}/(D_1)_{IC_{50}} + (D_2)_{IC_{50}}$, where $(D_1)_{IC_{50}}$ and $(D_2)_{IC_{50}}$ in the denominators are the drug concentrations for $D_1$ (drug 1) and $D_2$ (drug 2) alone that gives x% inhibition, whereas $(D_1)$ and $(D_2)$ in the numerators are the concentrations of drug 1 and drug 2 in combination that also inhibited x% (i.e. isoeffective). CI<1, C = 1, and C>1 indicate synergism, additive effect, and antagonism, respectively. Calculations were performed with the computer program CalcuSyn developed by Chou and Hayball [69].

Compounds

DHA was obtained from Chongqing Holley Wuling Mountain Pharmaceutical Co. Ltd, and artesunate from Guilin Pharmaceutical Co. Ltd.; artemisone, DSM265 [60], OZ277 [70], MMV390048 [71], OZ243 [72], OZ381 [73], P218 [59] and carbaOZ277 [74] were synthesized as previously described. Erlotinib and imatinib were supplied by LC-Laboratories (Woburn, MA, USA), cisplatin and vincristine by Sigma-Aldrich (Deisenhofen, Germany) and dasatinib by SanxinChemPharma (Shijiazhuang, China). Test compounds (antimalarials) were diluted in DMSO to a stock concentration of 26.7 mg/ml. Stock solutions for erlotinib, imatinib, cisplatin and dasatinib were prepared in DMSO at a concentration of 53.3 mM and vincristine at 67 μM.

Microarrays

RNA was extracted from cell lines using the mirVana® kit (Ambion) according to the manufacturer’s instructions. The RNA concentration was adjusted to 250 ng/μl. Biotinylated cRNA was prepared according to the standard Affymetrix protocol from total RNA (Expression Analysis Technical Manual, 2001, Affymetrix).

All microarray gene expression profiles were obtained using the Affymetrix HG-U133 Plus 2.0 GeneChip arrays. Hybridization was carried out according to standard Affymetrix protocol for 3’ IVT design and the GeneChips were scanned using the Hewlett-Packard GeneArray Scanner G2500A. All chips were assessed for

Figure 1. Molecular structures of compounds used in this study.
doi:10.1371/journal.pone.0082962.g001
quality using the “R” statistical computing environment and associated modules from Bioconductor with Percent Present cut-off of 35%, resulting in only a single array being rejected.

The CEL files were processed using RMAExpress (v. 1.04, Written by Ben Bolstad). The complete dataset (for 91 lines) was submitted to the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc = GSE51739) under accession number GSE51739. Following background adjust, quantile normalization and Median Polish summarization, expression values were exported in natural scale for further analysis. Affymetrix probe set annotations for the HG-U133_Plus_2 microarray were taken from build 33 (29 Oct 2012). Using the Excel® CORREL (Pearson’s) function, IC50 for each cell line were correlated with expression values, for a given compound and gene. In addition, probabilities for significance of these correlations were calculated using the Excel® formula =TDIST(ABS([Correlation]*/SQRT([nr of observations-2])]/SQRT(1-[Correlation]’2)), ([nr of observations-2]). In this function, “Correlation” refers to correlation efficient r as calculated earlier, while “nr of observations” refers to the number of cell lines tested.

In order to subdivide sets of genes associated with compound potency into clusters with similar function, Gene Ontology Biological Process assignments (http://www.geneontology.org/) were taken from the Affymetrix annotation file. Frequency scoring and data processing were performed in Excel®. Cluster analysis was performed using the Excel add-in Multibase 2013 package (Numerical Dynamics, Japan), using the Ward method (Cluster membership is assessed by calculating the total sum of squared deviations from the mean of a cluster).

Results

Testing of antimalarials for activity against cancer cell lines

Antimalarials representing five distinct target classes were tested in this study: (i) the three artemisinins artsunate, dihydroartesinin (Artenimol, DHA), and arteisone (BAY 44-9585), another semi-synthetic artemisinin derivative that presents an improved safety profile [57,75]; (ii) synthetic ozonides OZ439 and 277, synthetic peroxides with enhanced in vivo stability [76]; (iii) pyrimethamine and P218, established and second-generation DHFR (dihydrofolate reductase) inhibitors, respectively [59]; (iv) DSM265, a potent and selective triazolopyrimidine-based inhibitor of the enzyme dihydroorotate dehydrogenase (DHODH) which kills Plasmodium in vitro and in an in vivo mouse model [60]; (v) MMV390048, a 3,5-diaryl-2-aminopyridine/2-trifluoromethyl-pyridine that is hypothesized to kill Plasmodium by inhibiting one or more of the parasite’s kinases.

We included carbaOZ277, an inactive non-peroxide derivative of OZ 277 and OZ 381 and OZ 277, analogs of OZ277 and OZ439 (See Fig. 1 for their structures). As a positive control Paclitaxel, an established cancer drug, was included.

Fig. 2 lists the potencies as IC50 (µM) for the set of solid tumors and leukemic cell lines tested, and the origin (organs) of the lines. There is considerable variation both between compounds and, for a given compound, between cell lines tested. In addition to paclitaxel, strong potencies were seen for the three artemisinins, the two synthetic peroxides and the two DHFR inhibitors. By contrast, the DHODH and assumed kinase inhibitors and negative control compounds (OZ381, OZ721, carbaOZ277) lacked antitumor activity in these assays. This is the first demonstration of an anticancer activity of synthetic peroxides. While a single study has demonstrated that pyrimethamine induces apoptosis in melanoma cells [38], we here extend these findings to a much larger panel of cancer types. In addition by using a second, different but related DHFR inhibitor, the fingerprint of specific inhibition can be defined. In some cases, lines that were relatively insensitive to

Figure 2. IC50s (in µM) of various antimalarials for a panel of human tumor cell lines. DT, h doubling time (hours). doi:10.1371/journal.pone.0082962.g002
paclitaxel also showed resistance to the antimalarials, especially the hepatoma lines (e.g. HLE, SNU423, SNU739). By contrast, other lines that responded poorly to paclitaxel showed excellent sensitivity towards the antimalarials (e.g. gastric cancer IM9).

Effects of antimalarials on cancer cell expression profiles by microarray analysis

In order to group the compounds tested on the basis of their relative potencies in this cell line panel we performed a cluster analysis, with the compound’s IC$_{50}$ as input for the MultiBase analytical package from Numerical Dynamics. As shown in Fig. 3, this analysis most tightly clustered the two synthetic peroxides and the two DHFR inhibitors. Surprisingly, paclitaxel clustered with ART, DHA and Artemisone. While artemisinins and paclitaxel each kill cancer cells by inducing apoptosis, the former (artemisinins) are believed to do so through the intrinsic apoptotic pathway involving caspase 3 and 9, whereas paclitaxel exploits the caspase 8 pathway [77]. The similarity we observe here could

![Cluster Analysis](image)

**Figure 3. Cluster analysis for IC$_{50}$s of various antimalarials.** The IC$_{50}$ stands for the compound concentration where half-maximal inhibition is observed. doi:10.1371/journal.pone.0082962.g003

![Figure 4. Assessment of drug-drug interaction (determination according to Chou-Talalay); A purely additive effect results in C = 1; lower CI values reflect synergy (see Methods for details). doi:10.1371/journal.pone.0082962.g004](image)
reflect that some of the cancer lines we have used are susceptible to apoptosis induced by either pathway. The fact that the synthetic peroxides cluster separately as a group, and are clearly separated from the artemisinins is intriguing. It suggests that the synthetic peroxides kill cancer cells by more than one mechanism in spite of the fact that they share a characteristic peroxide pharmacophore with the artemisinins. This also suggests that the mechanisms whereby these human cell lines develop resistance to artemisinins and to the synthetic peroxides could be completely different.

Since we found that the different antimalarials tested displayed distinct characteristics potentially reflecting diverse mode of actions we decided to further explore these compounds for potential synergies with the established anticancer drugs erlotinib, imatinib, cisplatin, dasatinib and vincristine. Using a subset of cell lines, the antimalarials were tested at three different concentrations together with a gradient of these anticancer drugs. The assessment of drug-drug interaction was determined according to Chou-Talalay (see Methods) and the results are displayed in Fig. 4. Both OZ 277 and ART exercised significant synergy with vincristine (as reflected by a low IC value in the red-colored cells). Vincristine is known to polymerize microtubules, resulting in mitotic arrest in the metaphase, while earlier mitotic phases are unaffected [78]. By contrast, artemisinins are known to arrest cell division at a much earlier stage, namely the G1/G2 phase (eg. [56,79]). Thus, while both type of compounds eventually induce apoptosis, it is possible that cells that somehow escape from the G1/2 phase block are subsequently trapped in the metaphase due to the action of vincristine, resulting in a synergetic mode of action.

Erlotinib is an EGFR (Epidermal growth factor receptor) inhibitor that we found synergizes with ART in five out of eight lines tested, while dasatinib, a c-abl inhibitor, stimulated activity in three lines. Since kinase inhibitors typically target a set of related kinases it may be difficult to predict which lines may present the best synergistic response.

The checkered appearance of the Table in Fig. 2 reflects the modern view that cancers should be characterized by the molecular mechanism(s) that allows them to escape from proliferative controls, as opposed to a characterization by their organ of origin. In order to gain further insight in the molecular mechanisms that are associated with the compound’s potencies we performed a gene expression microarray analysis of nearly every cell line used in this panel. For each compound tested, we subsequently correlated its inhibitory pattern throughout the cell line panel with gene expression variation, calculating correlation coefficients and their associated probabilities for each gene in the array (see Methods). We thus obtained, for each compound, lists of genes whose expression pattern correlated (positively or negatively) with compound potencies. Examples of such correlations are shown in Fig. 5. While expression of tollip (two probe sets) in most cell lines was somewhat below our 500 cutoff, potency of DHA is negatively correlated with expression of this gene (see Supplemental S1), a kinase substrate implicated in breast cancer [80]. A
similar example is DUSP8, a gene whose promoter methylation predicts clinical outcome of ovarian cancer [81] and MAP-KAPK2, which is known to regulate invasion of bladder cancer [82]. In order to obtain an aggregate, functional view of these associated genes we listed, for each compound, all genes whose expression significantly (cutoff $p < 0.0005$) correlated with compound potency (positive and negative correlations were grouped). The corrected (Bonferroni) threshold for the 16,722 genes that we considered (those with expression signal $> 500$) would be at $p < 3.10^{-16}$; however, this type of correction has been criticized as conservative (eg [83]) and might in our case skew the subsequent aggregate analysis (see below), which is less sensitive to the false discovery rate. The lists of genes associated with each compound are provided as a Supplement (S1). Next, the functional annotations (from the Gene Ontology Biological Process assignments, eg “DNA repair”) for the genes in each list were catalogued, and their frequencies (top 30) were plotted in histograms (Fig. 6), along with the number of genes for each list; similar results were obtained using the GENECODIS package ([84], data not shown). As expected, regulatory genes involved in signal transduction, transcription and apoptosis were prominent but, given that different gene sets associated with the compounds, remarkably similar; presumably, different genes associated with the various compounds were binned in the same category, resulting in the apparent convergence when results are displayed in this “high-level” format. Previously, a similar study to ours was performed with artemisunate, but using a different cell line panel and microarray platform [85]. Interestingly, the top-ranked “resistance” gene in that study, SLC30A1, was also assigned by us as such (Data S1), and we found that expression of this gene was only significantly associated with ART ($p = 0.0026$), not the other compounds, emphasizing the unique character of each compound that we tested. SLC30A1 is a zinc efflux transporter; a Plasmodium orthologue exists with Swissprot accession Q8IBU1. One possibility that explains the confirmed link with ART resistance is that overexpression of zinc transporters protects from apoptosis [86]. The gene is also important in erythrocytes [87] and may affect the uptake of Fe²⁺ [88], which was shown to be a critical mediator of ART toxicity [89–92]. Another published study that evaluated ART and gene expression used the NCI60 cell line panel (from the National Institute for Cancer [64]). This study [90] identified the transferrin receptor (TRFC) as associated with ART resistance; in our study we also find this gene to be significantly associated with ART, artemisone and DHA ($p = 0.01$, 0.001 and 0.01, respectively) also as a “sensitivity gene” (i.e., higher expression results in greater compound sensitivity). From that study, we also confirmed the association of ART inhibition with ABC Transporter ABCB7 ($p = 0.01$).

Finally, a pathway analysis was performed to graphically display relationships between sets of genes in terms of the molecular partners they are known to interact with; using this type of analysis, separate sets of genes may reveal that they interact with a common “target” set of genes. However, an analysis using the VisANT package revealed no obvious relationships [93,94]; data not shown). One of the difficulties with this and similar packages (such as Ingenuity Pathway Analysis) is that “interactions” are derived from disparate observations: protein-protein interactions and enzyme-substrate relationship from different cell types and in various contexts. These relations are as yet incomplete and biased towards intensely studied and abundant (protein-protein interactions) partners.
We have evaluated five distinct classes of antimalarials for their potential use in cancer. Three of these, the artemisinins, the synthetic peroxides and DHFR (dihydrofolate reductase) inhibitors potently inhibited the growth of several human cancer lines. This high success rate may appear surprising, however, parasites and cancer cells share basic characteristics related to the metabolic requirements associated with their high proliferation rate.

Table 1. Human pharmacokinetics of antimalarials tested in this study.

| Compound (mg)       | Cmax      | AUC        | Source | Half-life |
|---------------------|-----------|------------|--------|-----------|
| OZ 439 (capsule)    | 50        | 17 ng/ml   | 102 ng.h/ml | [99]   |
|                     | 100       | 34 ng/ml   | 249 ng.h/ml | [99]   |
|                     | 200       | 102 ng/ml  | 890 ng.h/ml | [99]   |
|                     | 400       | 135 ng/ml  | 1,130 ng.h/ml | [99] |
|                     | 800       | 315 ng/ml  | 3,010 ng.h/ml | [99]   |
|                     | 1,200     | 701 ng/ml  | 6,530 ng.h/ml | [99]   |
| OZ 439 (dispersion) | 400       | 566 ng/ml  | 5,430 ng.h/ml | [99]   |
|                     | 800       | 917 ng/ml  | 9,630 ng.h/ml | [99]   |
|                     | 1,600     | 1,340 ng/ml| 17,500 ng.h/ml | [99] |
|                     | 800–1,200 | ~1,600 ng/ml|        | [99]   |
| OZ 277              | 50        | 8 ng/ml    | 40 ng.h/ml (0–8 h) | [101] |
|                     | 100       | 19 ng/ml   | 105 ng.h/ml | [101] |
|                     | 200       | 41 ng/ml   | 239 ng.h/ml | [101] |
|                     | 50        | 14 ng/ml   | 79 ng.h/ml (0–8 h) | [101] |
|                     | 100       | 25 ng/ml   | 152 ng.h/ml | [101] |
|                     | 200       | 68 ng/ml   | 408 ng.h/ml | [101] |
| ART/DHA             | 120 mg iv | 13,700–17,000 ng/ml | 876–1,038 ng.h/ml | [98] |
|                     | 120 mg iv; DHA readout | 1,500–2,760 ng/ml | 1,845–3,298 ng.h/ml | [98] |
|                     | 200 mg po | 67–119 ng/ml | 67–256 ng.h/ml | [98] |
|                     | 200 mg po, DHA readout | 654 ng/ml | 1,158–1,300 ng.h/ml | [98] |
|                     | 120 mg im | 884 ng/ml  | 999 ng.h/ml | [98] |
|                     | 120 mg im, DHA readout | 1,166 ng/ml | 2,474 ng.h/ml | [98] |
|                     | 120 mg ir | 448 ng/ml  | 796 ng.h/ml | [98] |
|                     | 120 mg ir, DHA readout | 219 ng/ml | 965 ng.h/ml | [98] |
|                     | 40 mg DHA | 39 ng/ml   | 98 ng.h/ml (infin.) |      |
|                     | 60 mg ART | 183 ng/ml  | 155 ng.h/ml (infin.) |      |
| Artemisone          | 10 mg po  | 40 ng/ml   | 30 ng.h/ml | [102]   |
|                     | 20 mg po  | 57 ng/ml   | 66 ng.h/ml | [102]   |
|                     | 30 mg po  | 51 ng/ml   | 72 ng.h/ml | [102]   |
|                     | 40 mg po  | 83 ng/ml   | 118 ng.h/ml | [102] |
|                     | 80 mg po  | 140 ng/ml  | 282 ng.h/ml | [102] |
| pyrimethamine       | 75 mg po  | 34,700–38,400 ng.h/ml |        | [103] |
|                     | 50 mg/d, 3 wk | 2,059 ng/ml | 41,800 ng/ml *h (24 h) | [100] |
|                     | 75 mg single dose | ~800 ng/ml | 106,065 ng *h/ml | [104] |
|                     | 25 mg single dose | 760 ng/ml | 76,000 ng *h/ml | [105] |
|                     | P218      | 15.8 uM (~6,257 ng/ml) |        | [59]   |

doi:10.1371/journal.pone.0082962.t001

Discussion

We have evaluated five distinct classes of antimalarials for their potential use in cancer. Three of these, the artemisinins, the synthetic peroxides and DHFR (dihydrofolate reductase) inhibitors...
parasitcidal and anticancer mechanisms of artemisinins have been extensively studied and are now well understood to be linked to an environment rich in free or heme-bound intracellular Fe$^{3+}$ and the induction of apoptosis [13]. Nevertheless there are still uncertainties over the relative importance of the direct generation of reactive oxygen species and more indirect consequences of artemisinins binding to mitochondrial heme and the induction of the intrinsic apoptotic pathway. Our surprising finding that the three artemisinins can functionally be clearly distinguished from the synthetic peroxides, in spite of the fact that they all share a common peroxide pharmacophore, strongly suggests that these molecules have multiple modes of action, not all of which are distributed equally among members of the two families. DHFR is required for the mitochondrial thymidylate biosynthesis pathway, a critical process in rapidly reproducing cells. While the antiproliferative properties of these antifolates had mostly been evaluated in rapidly proliferating T lymphocytes, their potential use in cancer was only recently identified [58]. The present study confirms and extends these findings: we include a novel parasite DHFR inhibitor P218, that displays similar anticancer properties as pyrimethamine, and the similarity of the changes in the gene expression profiles of cell lines inhibited by the two compounds suggests a similarity in the mode of action. One of the reasons for developing P218 is the existing resistance observed in field isolates of Plasmodium to pyrimethamine. The similarity in the profiles of cancer cell lines inhibited by the two compounds, suggests that P218 may also be considered in combination with pyrimethamine to prevent the emergence of DHFR resistant tumor cells. We found no significant activity for DHODH inhibitors. One explanation for this is the stringent species specificity of these drugs (which target the Plasmodium ortholog), since one DHODH inhibitor, brequinid, did show (modest) efficacy in cancer [95]. The overarching pragmatic rationale for evaluating the activity of these antimalarials against cancer tissue-derived cell lines is that these drugs have well-established, excellent safety profiles. Furthermore, as we have shown in this study, their unique modes of action present opportunities for combinatorial use with established cancer drugs. Artemisinins are known to stall mitosis at the G1/G2 phase, while pyrimethamine results in a block at the S-phase [58] and paclitaxel at the metaphase; possibly such considerations constitute a firmer basis for deciding on potential synergy between these drugs than a focus on the upstream pathways that these cancer cells utilize to drive proliferation. Furthermore, at least for the time being our descriptive capabilities (genetics and transcriptionomics) have greatly outpaced our integrative capacity, in other words, our ability to reduce these data into an understanding of a cancer cell’s specificity to cause disease. Either way, discovery of optimal combinatorial strategies remains therefore a matter of trial and error. In other words, while our data indicate that these compounds behave strikingly different, this knowledge cannot yet be translated into reliable predictions of how they should be used.

In order to assess the potential of these antimalarial compounds for a use in cancer chemotherapy it is also important to compare our data with their exposure in human subjects. Table 1 summarizes key pharmacokinetic properties for the active compounds used in this study, including $C_{\text{max}}$, AUC (Area Under the Curve) and plasma half-life. Unfortunately these values are typically presented as weight/ml; as a rule of thumb and for comparison with the data in Fig. 2, 1 µg/ml corresponds to 2–3 µM (for compounds with MW 500 and 333, resp.). As the Table shows, artemisinins generally have a short plasma half-life. They are considered prodrugs with rapid conversion into DHA, which has a somewhat longer half-life. Since we show that both ART and its derivative DHA have anticancer properties, we can predict that efficacy may be better than one would assume from looking at exposure of either substance alone. Among the different artemisinin derivatives, arsunate is best suited for iv administration, providing a means for achieving the highest exposure. It is therefore presently the treatment of choice for acute malaria [96,97] with peak exposure of 14–17 µg/ml when dosed at 120 mg iv [98], which is well above the IC$_{50}$ for many of the cell lines that we tested (Fig. 2), even though the potencies that we found in our anticancer assays are much weaker than what is found when testing against Plasmodium (eg in the nM range for arsunate [75]). The short half-life of some of the compounds that we tested may be a problem for a mode of action that would involve inhibition of angiogenesis [21], but this is probably not a problem for antiproliferative uses (as against Plasmodium); successful anticancer drugs such as cisplatin and taxol also have rather short half-lives (1.5–3.6 and 5.8 h respectively). For the synthetic peroxides, exposure of the advanced second generation drug candidate OZ 439 reaches 2.4 µg/ml, or 5.1 µM [99]. This concentration brings the compound very close to the IC$_{50}$ that we found for a number of cancer lines (Fig. 2). So far, this compound has been conservatively dosed in volunteers, commensurate with a use in malaria patients; however, its excellent safety profile could allow for significantly higher exposure in cancer patients. Also, in contrast to artemisinins the compound has excellent half-life properties, thus presenting considerable potential for clinical use. Finally, pyrimethamine generates an exposure of 2,059 ng/ml (>8 µM [100]), well above the IC$_{50}$ of the majority of cell lines that we tested (Fig. 2). While the exposure in humans for P218 is not yet known, our study suggests that this compound has the potential to address resistance against this category of drugs both in malaria and cancer.

In summary, we have established that three mechanistically different antimalarials show potent in vitro activity for a variety of cancer cell lines at concentrations that are established or reachable in patients. We also demonstrate evidence for potential synergy with established cancer drugs.

Given the well-established safety profile of these drugs, further evaluation of their potential in a clinical setting is recommended.

Supporting Information

Data S1  Correlation coefficients between gene expression and compound potency (cutoff p = 0.0005). The “Minimum p-value” refers to the lowest p-value for the set of compounds that a gene was examined for.

Author Contributions

Conceived and designed the experiments: TN CW RHvH IP GK. Performed the experiments: IP GK. Analyzed the data: RHvH TN CW IP GK. Contributed reagents/materials/analysis tools: RKG RC RKH MAP JLV YY. Wrote the paper: RHvH.

References

1. Krishna S, Bustamante L, Haynes RK, Staines HM (2008) Artemisinins: their growing importance in medicine. Trends Pharmacol Sci 29: 520–527.

2. Zhang CZ, Pan Y, Cao Y, Lai PB, Liu L, et al. (2012) Histone deacetylase inhibitors facilitate dihydroartemisinin-induced apoptosis in liver cancer in vitro and in vivo. PLoS One 7: e39870.
29. O'Neill PM, Barton VE, Ward SA (2010) The molecular mechanism of action of dihydroartemisinin. J Biol Chem 285: 30059–30067.
30. Wu J, Yu Y, Ma J, Zhang H, Zhang N, et al. (2012) Lp-P selectin modification to enhance delivery of dihydroartemisinin to the tumor microenvironment. PLoS One 7: e37222.
31. Chen X, Lin H, Wen J, Rong Q, Xu W, et al. (2012) Dihydroartemisinin suppresses cell proliferation, invasion, and angiogenesis in human glioma U87 cells. African Journal of Pharmacy and Pharmacology 6: 2433–2440.
32. Eichhorn T, Schlossig S, Hahn B, Wendler A, Mertens R, et al. (2010) Bioinformatic and experimental fishing for artemisinin-interacting proteins from human pancytopenic cancer cells. Mol Biol Rep 37: 1079–1086.
33. Chen H, Shi L, Yang X, Li S, Guo X, et al. (2010) Artesunate inhibiting angiogenesis induced by human myeloma RPMI8226 cells. Int J Hematol 92: 507–517.
34. Zhou HJ, Wang WQ, Wu GD, Lee J, Li A (2007) Artesunate inhibits angiogenesis and downregulates vascular endothelial growth factor expression in human metastatic melanoma cells. Vet Res 38: 131–152.
35. Wang J, Guo Y, Zhang BC, Chen ZT, Gao JF (2007) Induction of apoptosis and inhibition of cell migration and tube-like formation by dihydroartemisinin in murine lymphatic endothelial cells. Pharmacology 78: 207–218.
36. Chen HH, Zhou HJ, Wu GD, Lou XD (2004) Inhibitory effects of artemisinin on angiogenesis and on expressions of vascular endothelial growth factor and VEGF receptor KDR/flk-1. Phytomedicine 11: 79–84.
37. Huan-huan C, Li-Ly, Shang-Bin L (2004) Artesunate inhibits angiogenesis in human U87MG cells. Cancer Lett 211: 163–173.
38. Zhou C, Pan W, Wang XP, Chen TS (2012) Artesunate induces apoptosis via a Bak-mediated caspase-independent intrinsic pathway in human lung adenocarcinoma cells. J Cell Biochem 113: 377–387.
39. Hendrick R, Oumkazte T, Bauer KD, Freier F, Ruhel A, et al. (2010) Dihydroartemisinin induces apoptosis by a Bak-dependent intrinsic pathway. Mol Cancer Ther 9: 2947–2951.
40. Kim SJ, Kim MS, Lee JW, Lee CH, Yoo H, et al. (2006) Dihydroartemisinin enhances radiosensitivity of human glioma cells in vitro. J Cancer Res Clin Oncol 132: 129–135.
41. Singh NP, Lai HC (2004) Artemisinin induces apoptosis in human cancer cells. Anticancer Res 24: 2277–2281.
42. Efferth T, Benakis A, Romero MR, Tomicic M, Rauh R, et al. (2004) Enhancement of cytotoxicity of artemisinins toward cancer cells by ferrous ions. Free Radic Biol Med 37: 98–1009.
43. Pedada D, Phillips T, Lin C, Gano SE (2002) Transferrin overcomes drug resistance to artemisinin in human small-cell lung carcinoma cells. Cancer Lett 179: 151–156.
44. Lai H, Singh NP (1995) Selective cancer cell cytotoxicity from exposure to artemisinin and halotetramycin. Cancer Lett 91: 41–46.
45. Daniels TR, Delgado T, Helguera G, Pentchel ML (2006) The transferrin receptor part II: targeted delivery of therapeutic agents into cancer cells. Clin Immunol 121: 159–176.
46. Daniels TR, Delgado T, Rodriguez JA, Helguera G, Pentchel ML (2006) The transferrin receptor part III: Biology and targeting with cytotoxic antibodies for the treatment of cancer. Clin Immunol 121: 148–158.
47. Zheng QP, Zhang PZ (2011) Artesunate mitigates proliferation of tumor cells by alkylating hemoglobin and inhibiting tubulin synthesis. Nutr Cancer 63: 110–112.
48. Zhang Q, Zeng XS, Zeng YB, Wang LF, Wei WP, Lu YF, Yang B (2009) Dihydroartemisinin potentiates the cytotoxic effect of temozolomide in rat C6 glioma cells. Pharmacology 82: 1–9.
49. Efferth T, Giasi M, Moelling A, Krammer PH, Li-Weber M (2007) Artesunate serves as a general anti-proliferation target. PLoS One 2: e693.
50. Kong R, Jia G, Chen XZ, Wang YW, Ma M, et al. (2012) Dihydroartemisinin enhances Apo2L/TRAIL-mediated apoptosis in pancreatic cancer cells via ROS-mediated up-regulation of death receptor 5. PLoS One 7: e37222.
51. Li JI, Chen SM, Ding J, Meng LH (2012) Characterization of dihydroartemisinin-resistant colon carcinoma HCT116/R cell line. Mol Cell Biochem 360: 329–337.
52. Efferth T, Herrmann F, Tahmazi A, Wink M (2007) Cytotoxic activity of secondary metabolites derived from Artemisia annua L toward cancer cells in comparison to its designated active constituent artemisinin. Pharmacognosy 16: 769–770.
53. Antonssen L, Efferth T, Ahlburg A, Pfeifer U (2006) Microarray expression profile of artemisinin-related genes predict tumor cell response to artemisinins. Pharmacogenomics J 6: 269–276.
54. Efferth T, Dunstan H, Sauerbrey A, Miyachi H, Chitambar CR (2001) The anti-malarial artemisinin is also active against cancer. Int J Oncol 18: 767–773.
55. Zhang XQ, Logemann K, Ruck RK, Yagman B, Pettit JT, Reckeweg K, Heilmann HD, et al. (2006) Artemisinin—A highly active antimalarial drug of the artemisinin class. Angew Chem Int Ed Engl 45: 2082–2081.
56. Giannamori AM, Maselli A, Casagrande A, Gambardella L, Gallina A, et al. (2004) Pyrvinium methobromide enhances cytotoxicity of melanoma cells via a caspase and camptothecin double-edged mechanism. Cancer Res 64: 5291–5300.
57. Yuhtavong Y, Tarnchompoo B, Vilaivan T, Chitnumsub P, Kamchonwongpaisan S, et al. (2012) Malarial dihydrofolate reductase as a paradigm for drug development against a resistance-compromised target. Proc Natl Acad Sci U S A 109: 16623–16628.
58. Cotecon JM, Marco M, Esquivias J, Deng X, White KL, et al. (2011) Structure-guided lead optimization of triazolopyrimidine-ring substituents identifies...
plasmid Potent evaluations of Plasmodium falciparum dihydroorotate dehydrogenase inhibitors with clinical candidate potential. J Med Chem 54: 5490–5561.
61. Roth T, Burger AM, Denger W, Willmann H, Fiebig HH (1999) Human tumor cell lines demonstrating the characteristics of patient tumors as useful models for anticancer drug screening. In: Fiebig HH, Burger AM, editors. Relevance of Tumor Models for Anticancer Drug Development, Contrib Oncol pp. 145–156.
62. Fiebig HH, Winterhalter B, Berger DP, Lohe GW (1989) Combined in vitro/in vivo test procedure with human tumor xenografts for anticancer drug development. Strahlenther Onkol 165: 522–524.
63. Fiebig HH, Denger WA, Roth T (1999) Human tumor xenografts: Predictivity, characterization, and discovery of new anticancer agents. In: Fiebig HH, Burger AM, editors. Relevance of Tumor Models for Anticancer Drug Development Contrib Oncol pp. 29–50.
64. Shoemaker RH (2006) The NCI60 human tumour cell line anticancer drug screen. Nat Rev Cancer 6: 813–823.
65. Masters JR, Thomson JA, Daly-Barns B, Reid YA, Dirks WG, et al. (2001) Short tandem repeat profiling provides an international reference standard for authentication of human cell lines. ALTEX 23: 102–109.
66. Denger WA, Schulte J, Berger DP, Mertelsmann R, Fiebig HH (1995) Development of a propidium iodide fluorescence assay for proliferation and cytotoxicity assays. Anticancer Drugs 6: 522–532.
67. Chou TC (1991) The median-effect principle and the combination index for quantitation of synergism and antagonism. In: Chou TC, Rideout DC, editors. Synergism and antagonism in chemotherapy. San Diego: Academic Press. pp. 61–102.
68. Chou TC, Hayball MP (1996) Calcsyn for Windows. Software for Dose Effect Analysis. Cambridge: Biosoft Copyright 1996–2005 (Calcsyn 2).
69. Vemurafenib, Arbe-Barnes S, Brun R, Charman SA, Chiu FC, et al. (2004) Identification of an antimarial synthetic trioxolane drug development candidate. Nature 430: 900–904.
70. Younis Y, Douelle F, Feng TS, Gonzalez Cabrera D, Le Manach C, et al. (2011) OZ277, OZ439, and Their 1,2-Dioxolane, 1,2,4-Trioxane, and 1,2,4,5-Tetroxane Isosteres. J Med Chem.
71. Hu Z, Hou J, Huang H, Wang Y, Chang YC, Huang CL, et al. (2009) OZ439: 3,5-Diaryl-2-aminopyridines as a novel class of orally active antimalarial agent with potent Plasmodium falciparum dihydroorotate inhibitory properties. Antimicrob Agents Chemother 53: 668–676.
72. Zhang X, Li A (2008) Dihydroartemisinin induces apoptosis in HL-60 leukemia cells dependent of iron and p38 mitogen-activated protein kinase activation but independent of reactive oxygen species. Cancer Biol Ther 7: 1017–1023.
73. Zhou HJ, Wang Z, Li A (2008) Dihydroartemisinin induces apoptosis in human leukemia cells HL60 via downregulation of transferrin receptor expression. Anticancer Drugs 19: 247–253.
74. M Zhang L, Alker A, Ruf A, Wang X, Chiu FC, et al. (2008) Characterization of novel tyrosine kinase substrates during breast cancer development. Mol Cell Proteomics 6: 2072–2087.
75. Huang XJ, Wang Z, Li A (2008) Dihydroartemisinin exerts cytotoxic effects and inhibits hypoxia inducible factor-alpha activation in C6 glioma cells. J Pharm Pharmacol 59: 849–856.
76. Younis Y, Douelle F, Feng TS, Gonzalez Cabrera D, Le Manach C, et al. (2011) Antimalarial activities and ADME Profiles of Ozonides (1,2,4-trioxolanes) synthesized by the two major CYP3A4 metabolites of ozonide (1,2,4-trioxolane) OZ277. Bioorg Med Chem Lett 18: 1555–1558.
77. Jacobson JM, Davidian M, Rainey PM, Hafner R, Raasch RH, et al. (1996) Artesunate versus quinine in the treatment of severe falciparum malaria: a randomised trial. Lancet 349: 717–725.
78. Dondorp AM, Fanello CI, Hendriksen IC, Gomes E, Seni A, et al. (2010) Artesunate versus quinine in the treatment of severe falciparum malaria in African children (AQUAMAT): an open-label, randomised trial. Lancet 376: 1647–1657.
79. Zhou HJ, Wang Z, Li A (2008) Dihydroartemisinin induces apoptosis in human leukemia cells HL60 via downregulation of transferrin receptor expression. Anticancer Drugs 19: 247–253.
80. Zhou HJ, Wang Z, Li A (2008) Dihydroartemisinin induces apoptosis in human leukemia cells HL60 via downregulation of transferrin receptor expression. Anticancer Drugs 19: 247–253.
81. Younis Y, Douelle F, Feng TS, Gonzalez Cabrera D, Le Manach C, et al. (2011) OZ277, OZ439, and Their 1,2-Dioxolane, 1,2,4-Trioxane, and 1,2,4,5-Tetroxane Isosteres. J Med Chem.
82. Kodry R, Stewart D, DeForni M, Moore M, Dallaire B, et al. (1993) Multicenter phase II study of brequinor sodium in patients with advanced breast cancer. Am J Clin Oncol 16: 526–528.
83. Moehle JJ, Duparc S, Borghini-Fulcher I, Jung D, Shin CS, et al. (2011) Review of the clinical pharmacokinetics of artesunate and its active metabolite dihydroartemisinin following intravenous, intramuscular, oral or rectal administration. Malar J 10: 263.
84. Zhou HJ, Wang Z, Li A (2008) Dihydroartemisinin induces apoptosis in human leukemia cells HL60 via downregulation of transferrin receptor expression. Anticancer Drugs 19: 247–253.
85. Morais CA, Dupar S, Borghini-Fulcher I, Jung D, Shin CS, et al. (2011) Review of the clinical pharmacokinetics of artesunate and its active metabolite dihydroartemisinin following intravenous, intramuscular, oral or rectal administration. Malar J 10: 263.