Title: In vitro study of the anti-cancer effects of artemisone alone or in combination with other chemotherapeutic agents

Authors: Andrew M. Gravett[1], Wai M. Liu[1], Sanjeev Krishna[2], Wing-Chi Chan[3], Richard K. Haynes[3], Natalie Wilson[1] and Angus G. Dalgleish[1]

Affiliation: [1]Department of Oncology, Division of Cellular and Molecular Medicine, St George's, University of London, UK.

[2]Centre for Infection, Division of Cellular and Molecular Medicine, St George's, University of London, UK.

[3]Department of Chemistry, Open Laboratory of Chemical Biology, Institute of Molecular Technology for Drug Discovery and Synthesis, The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong (PR China)

Correspondence: Andrew M. Gravett

Cellular and Molecular Medicine

St George's University of London

2nd Floor, Jenner Wing

London, SW17 0RE

T: +44 020 8725 5307

E: agravett@sgul.ac.uk

Keywords: Artemisinin, Artemisone, cancer, cell-cycle, combination.

Abstract

Purpose: Artemisinins are now established drugs for treatment of malaria. These agents have been shown to possess impressive anti-cancer properties. We have investigated the role of artemisone (ATM), a novel derivative of artemisinin (ART) in a cancer setting both alone and in combination with established chemotherapeutic agents. **Methods**: The anti-proliferative effects of ART and ATM were tested on a panel of human cancer cells *in vitro* using the methylthiazoletetrazolium assay, and the effect on cell cycling established by flow cytometry. Immunoblot analyses were performed to determine effects at the molecular level. Finally, ART and ATM were combined with the common anti-cancer agents oxaliplatin, gemcitabine and thalidomide. **Results**: ART and ATM caused dose dependent decreases in cell number. ATM was consistently superior to ART, with IC50s significantly lower in the former. Neither drug caused significant changes to the cell viability (%viable cells >95%), but arrested cell cycling. Blockade was either exclusively at the level of G1, or at all phases of the cell cycle, and associated with reductions in cyclin D1, CDK4 and pRb. Combination studies showed the anti-proliferative effect of ATM was often enhanced by addition of the other drugs, whilst ART exhibited antagonistic properties. **Conclusions**: ART and ATM are active in cancer cell lines, with ATM displaying the greater anti-proliferative effect when used alone. ATM also enhances the effects of the above drugs, with ART being less likely to improve activities. Taken together, ATM should be thought of as the ART-derived compound next in line for further study.

Introduction

Artemisinin (ART) is a natural trioxane that is isolated from *Artemisia annua L,* (qinghao or sweet wormwood). Historically qinghao possesses anti-pathogenic qualities, and has been used traditionally in China as a treatment for a variety of ailments. It was only in the early 1970s that the active principle, qinghaosu, or ART was first isolated and its anti-malarial properties first reported [1]. The parent ART has been structurally modified to provide various derivatives, known collectively as artemisinins. These are amongst the most potent anti-malarial agents known. The mechanism of action of artemisinins remains controversial [2, 3] but they retain activity against parasites that have become resistant to anti-malarials such as such as chloroquine and pyrimethamine [4]. Furthermore, artemisinins can be faster acting than other drugs malarial treatments [5, 6]. These impressive qualities have resulted in the World Health Organisation recommending that all anti-malarials be combined with an ART component when used in first line treatment [7]. One newer example of an artemisinin derivative is artemisone (ATM) which has emerged as a therapeutic candidate and possesses sustained activity in plasma compared to other derivatives. It is significantly more active against *Plasmodium falciparum* than other derivatives [4, 8, 9], has almost negligible toxicity [10], and is prepared in a three step process from the parent ART.

In recent years the artemisinins have also been shown to have anti-cancer properties, through their ability to reduce cell number in a variety of solid tumours *in vitro* [11-14] and in *ex vivo* animal models [15, 16]. Activity has also been seen in humans [17, 18], and a recent phase II study in patients with lung cancer reported

artemisinin combinations could extend short-term survival and time-to-progression rates [19]. Studies have identified several potential mechanisms by which the artemisinins can act against cancer cells. They have been shown to be anti-proliferative through action on key cell cycle regulatory proteins such as $p21^{waf1cip1}$ and cyclin D1 [20]; pro-apoptotic by manipulating the Bax:Bcl-2 rheostat [21,22]; anti-angiogenic by targeting vascular endothelial growth factor [23,24]; and anti-migratory through their effects on αVβ3 integrins [25]. The multi-modal character of these anti-malarial drugs, allied to their low host toxicity even at high doses [26, 27], reinforces the priority for them to be developed as a novel anti-cancer agents.

It has been known for some time that the anti-malarial efficacy of the artemisinins is significantly enhanced when used in combination with other agents, which is an approach that may be beneficial in cancer. Indeed, artemisinins have already shown some synergy with common chemotherapeutic agents [28]. Furthermore, cancer cells can become ‘addicted’ to certain pathways, which can ultimately lead to drug resistance when single agents that target specific pathways are used [29]. Consequently, using drugs that display a wider target-window, such as the artemisinins, in combination with more established cytotoxic drugs may improve overall activity. Therefore, the aims of the current study were to investigate the anti-proliferative effect of ATM in a panel of human cancer cell lines *in vitro*, and compare this with the activity of the parent ART. Additionally, we also investigated the possible benefits of combining these artemisinins with conventional chemotherapeutic agents.

Materials and methods

Drugs

Artemisinin was obtained from Sigma Ltd., Dorset, UK. Artemisone was synthesized and purified as previously described [4]. Each was reconstituted at 10mM in dimethyl sulfoxide (DMSO) and stored at -20^{o}C. Gemcitabine (GEM: Eli Lilly, Pharmacy, St George's Hospital, UK), oxaliplatin (OXP: Sigma) and thalidomide (THAL: Celgene Corp., Summit, NJ, USA) were all reconstituted in phosphate buffered saline (PBS) and stored at -20^{o}C.

Cell culture

The human cancer cell lines; MCF7 (breast), HCT116 and SW480 (colon) (Cancer Research UK, London, UK), KM and MJT3 (melanoma) (in house), PANC-1 and MIAPaCa (pancreas) (European Collection of Cell Cultures, Salisbury, UK) were grown in either DMEM (Sigma) or RPMI (Sigma) media supplemented with 10% foetal bovine serum, 2mM L-glutamine, 1% penicillin/streptomycin. All cell lines were incubated in a humidified atmosphere with 5% CO_2 in air at 37°C, and only cells with a passage number <10 were used in the experiments.

Proliferation assays

To study the effect of ATM and ART on cell growth, cells growing exponentially were added to 96-well plates at a density of 3 x 10^4 /well. Drugs (0.1 – 100μM) were then added to the wells, ensuring an equal volume of 200μL across the plate. Cell number/proliferation was measured at 72 hr using a standard methylthiazoletetrazolium (MTT)-based assay without modifications. Briefly, MTT (Sigma) was added to each well to give a working concentration of 0.4mg/ml, and

plates returned to the incubator for a further 1 hr. After this time, the medium was aspirated off, 200µL of DMSO was then added to each well, and plates agitated gently for 5 min before measuring optical density at 540 nm in each well.

Flow cytometric analysis of the cell cycle

Cells were cultured with equi-active concentrations (~IC25) of ART or ATM for 72 hrs, before fixing in 70% (v/v) ethanol in PBS. Following an incubation period of at least 30 mins, cells were washed and re-suspended in a DNA staining solution (1mg/mL propidium iodide and 1ng/mL RNAse A) (both Sigma). Acquisition of data was performed within 1 hr using a Becton Dickinson FACSCalibur (BD Biosciences), and gating was employed to remove doublet artefacts and to discriminate cells from debris. Ten thousand cells were analysed, and the percentages of cells in G1, S and G2/M phases were determined using the cell cycle analysis program WinMDI CellQuest v2.9 (http://facs.scripps.edu/software.html).

Immunoblotting analysis

Cells were harvested and total cellular protein was solubilised in lysis buffer (New England Biolabs, Hitchin, UK) and resolved by Tris-glycine electrophoresis using a 4-12% bis-tris gradient-gel according to the method of Laemmli [30]. Following transfer of proteins to nitrocellulose membranes (0.45 µm), blocking was performed in 5% (w/v) non-fat milk in TTBS [0.5% (v/v) Tween-20 in TBS (50mM Tris, 150 mM NaCl, pH 8.0)]. Primary antibody probing was performed with anti-p53, anti-$p21^{waf1/cip}$, anti-Bax, anti-CDK4, anti-cyclin D1, ant-CDK1, anti-cyclin B1 or anti-pRb. All primary antibodies were obtained from New England Biolabs (Hitchin, UK) and used at a dilution of 1:1,000. Anti-GAPDH was used as a loading control

(1:2,000 – New England). Following five washing steps in TTBS, horseradish peroxidase-conjugated anti-species IgG1 was used as the secondary antibody (Amersham Biosciences Ltd., Little Chalfont, UK). Bands were visualised using SuperSignal West Pico chemiluminescent substrate (Pierce).

Combination studies: fixing the ratio of the concentration of the drugs

Our combination studies followed an approach previously described [31]. HCT116, SW480 and MCF7 cells (5 x 10^4 /well) growing exponentially were reset in fresh culture medium and aliquoted into 96-well plates. ATM or ART was combined with OXP at an equal ratio of their respective IC50 (eg. ½x IC50 of ART was combined with ½x IC50 of OXP). IC50 for OXP was 2μM for HCT116 and SW480 cells and 5μM for MCF7. Cells were incubated for 72 hr in a humidified atmosphere with 5% CO_2 in air at 37°C. Cell number was assessed by the MTT assay as described previously. The activities of drug combinations were established by comparing optical density readings from the treated wells with the control wells with no drug treatment, and data were expressed as a fraction unaffected (FU). The natures of drug-drug interactions were then assessed by calculating a combination index (CI) by using the median-effect equation [32], where CI-values of 1 indicated additivity; CI<1 indicated synergy and CI>1 indicated antagonism.

Combination studies: fixing the concentration of one drug

Median-effect analysis of combination requires the extrapolation of an IC50 value. Where this was not possible, we explored the effect of combining drugs by fixing the concentration of one drug (modulating agent) and testing its ability to influence the activity of the drug partner. Cells (5 x 10^4 /well) growing exponentially were reset in

fresh culture medium and aliquoted into 96-well plates. ART or ATM were diluted in growth medium and added to the plates in a range of drug concentrations to allow determination of IC50. The effect on these IC50 values of co-culture with a sub-optimal concentration (~IC10) of GEM (2nM) or THAL (1mM) was then tested. Cell numbers at 72 hr were assessed by the MTT assay as previously described. This enabled the assessment of the nature of any drug-drug interaction by comparing the IC50 for ATM and ART in the presence and absence of the combinatorial drug partner.

Results

ATM and ART reduce cell number

Generally, concentration-dependent decreases in cell number were seen in the cell lines when cultured with ART and ATM. The only exceptions to this were that PANC-1 cell number was unaffected by either of the drugs, and MJT3 cell number was unchanged during culture with ART (Figure 1). MCF7 cells were most sensitive with calculated concentrations required to reduce cell number by 50% (IC50) for ART and ATM of 44μM and 0.5μM respectively. Generally, ATM was more active than ART, which was most clearly highlighted when comparing the IC50 values (Table I). Of particular note, in MJT3, where ART had no effect, ATM significantly reduced cell number. Similarly, HCT116 and SW480 cells were around 30-fold more sensitive to ATM than to ART. These reductions in cell numbers were not associated with increased cell death, as assessed by trypan blue dye exclusion, but cell counts using this method did confirm the reduction in cell number seen with the MTT assay.

ATM and ART induce cell cycle blockade

Flow cytometric analyses revealed there was no increase in the apoptotic/sub-G1 phase of the cell cycle of HCT116, SW480 or MCF7 after culturing with ART or ATM (Figure 2). However, there were cell cycle changes that were particular to each drug and cell line. Treating HCT116 cells with either ART or ATM did not alter the distribution of cells within the G1, S and G2 phases, which taken with the results of the proliferation assays, suggested a general blockade at all phases (Figure 2). A similar cell cycle profile was seen in SW480 cells cultured with ATM. However, culturing SW480 cells with ART resulted in a significant decrease in the amount of cells in G1 (63 ± 2.6% vs. 53 ± 6.1% in untreated cells; $p<0.05$) and concomitant increases in the phases downstream of G1. This effect was reversed in MCF7 cells, with ATM causing a G1-selective blockade (83 ± 5.5% vs. 63 ± 4.4% in untreated cells; $p=0.015$), but a general arrest in all phases after culturing with ART (Figure 2). ATM was also used at higher doses (~IC50), in all of the cell lines. However, this increase in concentration did not cause any changes in cell cycle profile compared to those cells treated with IC25 ATM. (data not shown)

ART and ATM affect proteins that regulate cell cycling

Whole cell lysates from cell lines cultured with ART or ATM were immunoprobed for the expression of key cell cycle regulatory proteins. Generally, treatment with either drug reduced the levels of cyclin D1 and its associated CDK 4 (Figure 3). Although the ATM and ART were used at equi-active concentrations (~IC25), the former agent reduced these two cell cycle regulatory proteins to a greater extent. Reductions in cyclin D1 and CDK4 correlated with reduced pRb

expression, which was lower in ATM treated cells. Levels of CDK inhibitor $p21^{waf1/cip1}$ showed modest changes in all the cell lines, with increasing levels in HCT116 cells and small decreases in SW480 and MCF7. Along with the changes seen in the levels of G1/S transition proteins CDK4 and cyclin D1, there was also a parallel reduction seen with G2/M transition proteins CDK1 and cyclin B1 in HCT116 and SW480 cell lines.

ART and ATM can enhance the effects of some chemotherapy

The benefits of combining ART and ATM with common chemotherapeutics were explored using a number of models. Benefit was indicated either by low CI-values or by decreases in IC50-values. Results showed ATM to be a better than ART when used in combination with OXP or GEM. Whilst combining ART with OXP resulted in CI-values in HCT116, SW480 and MCF7 of 3.3 ± 0.57, 2.3 ± 0.13 and 2.7 ± 1.3 respectively, which were significantly different from a CI-value of 1 ($p<0.05$ in all cases), combining ATM with OXP results in an additive interaction (Figure 4). Similar patterns to these were seen between GEM and ART/ATM, with ART interfering with the activity of GEM and ATM having no significant effect. Combinations with THAL resulted in no significant interaction. (Figure 4).

Discussion

This study was undertaken to investigate the anti-cancer properties of ATM, a novel and potent artemisinin derivative, in a panel of cell lines. We had a particular interest in assessing effects on cell proliferation and cell cycling, and in comparing these effects with those of ART. Additionally, the activities of these agents when used in combination with existing chemotherapeutics were also assessed. We showed that ATM was more active against cancer cell lines than ART both as a single agent and as a combination partner, and that the effects were through disruptions to the cell cycle.

The first part of our investigation established, by MTT analysis and trypan blue cell counts, that both ART and ATM reduced cell numbers. However, the effect of ATM was much more profound, in that IC50 values for ATM were considerably lower than those for ART in all cell lines. IC50s were similar to those published previously [3] with other cell lines and were within the range of clinically achievable peak plasma concentrations reported for other artemisinins [33]. The increased activity between ATM and ART was most prevalent in the MCF7 breast cancer cell line, where IC50 was around 80-fold less for ATM than its precursor ART. Conversely, neither drug had an impact on the p53-mutant non resistant PANC-1 cell line, although it remains to be seen whether this resistance could be overcome by strategic combinations involving other agents.

One of our most interesting findings was that ATM could significantly deplete cell numbers in lines seemingly resistant to ART, as was the case with MJT3 cells. Our results also showed that the ability of ATM to reduce cell numbers *in vitro* was much greater than that of ART both in magnitude and breadth, with potent activity in breast,

colon, pancreas and melanoma cancer lines. ATM has previously been shown to be consistently more active against malarial parasites than other artemisinins [9] A possible reason for this may be the superior bioavailability of ATM, which results in higher levels in plasma and sustained activity. Taken together, these results suggest that ATM may be more successful against cancer in *in vivo* studies than the previously studied artemisinins.

Trypan blue dye exclusion analyses conducted in parallel to the MTT assay showed both ART and ATM did not affect cell viability (data not shown), which suggested their effects were cytostatic rather than cytotoxic. For this reason we investigated the impact of the drugs on cell cycle distribution. The results revealed an absence of cell death (sub-G1 population) in cultures with ART or ATM, which was further supported by immunoblotting where no increase in the pro-apoptotic protein Bax was observed. Changes in the cell cycle were observed after treatment with the agents; specifically, cell cycle arrest as reported previously [20, 34-36]. Our results showed that the compounds generally induced a simultaneous arrest at all phases of the cell cycle. However, in the case of MCF7 cells treated with ATM an accumulation of cells within the G1 phase was observed indicating a block exclusively at G1/S interface. Conversely, in the response of SW480 cells to ART there was a significant decrease in the number of cells in G1 and a trend towards increased numbers in S and G2, signifying a cell cycle block downstream of G1. After seeing an additive effect when using ATM and OXP in combination against tumour cells, cell cycle analysis was performed. However, there was no significant change in the levels of apoptosis or cell cycle blockade when using ATM and OXP together when compared to using the single agents alone (data not shown).

Having seen alterations in the number of cells at the differing points in the cell cycle after culture with ART and ATM, we next investigated the molecular basis of these changes using protein immunoblots. Cell cycle transition is tightly regulated by a series of proteins that coordinate transitions between phases. Of these, we focussed on those regulating G1-to-S passage as they have been shown to be affected by artemisinins (36). The protein that directly controls this is pRb. A consequence of losing this protein is a block in G1. Our results revealed a dramatic reduction in levels of pRb in each cell line in response to ATM, and in MCF7 cells only, after culture with ART. This decrease in pRb was always associated with a decrease in the levels of its regulatory proteins CDK4 and cyclin D1, which maybe mediated by sp1 interaction [37]. Notably, pRb was increased in SW480 cells cultured with ART, which correlated with the decrease in the number of cells in the G1. The diverse effects of ART and ATM support the notion that artemisinins may have multiple modes of action. Indeed, culturing HCT116 and SW480 cells with ART or ATM caused significant reductions in the G2 regulatory protein CDK1, highlighting an additional blockade at G2/M transition, which supports our original idea that these agents may induce a global cell cycle arrest in HCT116 cells and a block downstream of G1 in SW480 cells. However, CDK1 levels increased in MCF7 cells after culturing with ART and ATM, possibly as a result of a prevalent G1 arrest [38].

.

Previously it has been shown that artemisinins can affect the cell cycle in a p53-dependent or independent manner [34]. Our data supports this in that cytostasis was achieved with or without up-regulation of p53 and $p21^{waf1/cip}$. Interestingly, ART and ATM had no effect on the p53 mutant cell line PANC-1. It maybe that an active form

of p53 is required for the cytostatic mechanism of ATM, however, other studies have shown that the artemisinins can exert their effects on p53 mutant [20] and p53 knock-out [34] cell lines. More importantly, the DNA damaging qualities of this class of agent has yet to be fully elucidated, with a wealth of data highlighting both genotoxic and non-genotoxic effects [39]. It is important to clarify this point and connect together genotoxicity and a p53 response. This is currently ongoing and forms the basis of another study. However, obstruction of the cell cycle can be considered to be one of the most effective strategies in the control of tumour growth, and with the effects observed in the present study ATM may become an important compound in the armoury of agents available to achieve this.

Cancers are characterised by multiple genetic defects, which may reduce the efficacy of single agent chemotherapy. Therefore, by using combination therapies to hit either diverse pathways or mutually exclusive points on the same pathway we can reduce the chance of cancer cells evading treatment. This underlies the idea of combination strategies where drugs are used simultaneously to achieve an effect that is greater than the sum of its parts [29]. However, care needs to be taken when choosing combinations to minimise undesired interactions. Evidence from other studies suggests that some artemisinins may potentiate other, more established treatments [40-42]. We therefore tested the ability of ART and ATM to sensitise HCT116, SW480 and MCF7 cells to OXP and GEM. The drugs selected are established treatments for colon or breast cancer. Also, these drugs affect cell cycle and influence p53 or $p21^{waf1/cip}$ [43, 44]. THAL was selected on the basis of targeting $p21^{waf1/cip}$ [45].

There are a number of models used to investigate the benefits of combining drugs. One method previously used by our group employs the median-effect equation to generate CI-values. These values give an indication of the 'worth' of a combination with CI>1 signifying antagonism and CI<1 suggesting enhancement of effect between the compounds. These calculations rely on sigmoidal growth inhibition curves being generated for each compound; however, the response curves for GEM and THAL did not meet this criterion, and hence, the median effect equation could not be used. Consequently, we used small sub-active concentrations of each of the drugs in an attempt to modulate the effect of ART and ATM according to a model described previously [31].

Combinations involving artemisinins have been studied *in vitro* and *in vivo* in *v*arious cancer types [20, 42]. Generally, combining artemisinins with other drugs have increased the anti-tumour effect; however, ART has been shown to hinder some treatments via up-regulation of calmodulin signalling [46]. The present study suggests that ATM has a much better combination portfolio than ART. Generally, ART-combinations were mostly antagonistic, whilst, although no true synergistic interactions occurred; non-antagonistic effects were prevalent when ATM was used. Specifically, the CI-values for combinations with ATM and OXP were significantly lower than for those for ART and OXP. Similar results were seen when the artemisinins were combined with GEM. Therefore, as ART has previously been shown to be advantageous in combinational therapies, our results suggest that ATM has the potential to be more so.

In conclusion, the artemisinins are a class of agent approved for use against malarial parasites that are resistant to most other treatments. As with malaria, the exact mechanism by which these compounds exert their anti-cancer effects remains undefined, but they can disrupt cancer cells by inducing apoptosis and blocking the cell cycle, so they should hinder disease progression, and thus, become important players in cancer treatment. This study is the first demonstration of anti-tumour efficacy of ATM. In a similar fashion to its effects in malaria, we have shown that ATM has more potent activity against cancer than other artemisinins. Presently, artemisinins are being tested in phase II clinical trials against cancer, and it seems rational for a closely related compound with greater activity, together with a lower toxicity to also be considered for similar studies. However, it remains to be seen whether ATM retains its high activity against cancerous cells *in vivo.* Overall, the possibilities of ATM are very promising and it should be thought of as the next artemisinin derivative to warrant further investigation in a cancer setting.

Acknowledgements

The authors would like to thank Dr John Copier for helpful discussions and critical reading of the manuscript. The authors would also like to thank Celgene Corps. for supplying thalidomide. Work at the Hong Kong University of Science and Technology was carried out in the Open Laboratory of Chemical Biology of the Institute of Molecular Technology for Drug Discovery and Synthesis with financial support from the Government of the HKSAR University Grants Committee Areas of Excellence Fund, Projects No. AoE P/10-01/01-02-I, AOE/P-10/01-2-II and the

University Grants Council Grants No. HKUST 6493/06M and 600507. Professor Krishna is funded by the European Commission projects ANTIMAL (Grant No. 018834) and MALSIG (Grant No. 223044). This work was funded by the cancer vaccine institute (www.cancervaccine.org.uk).

Disclosures

The authors have no conflicts of interest to declare.

References

1. D.L.Klayman (1985). Qinghaosu (artemisinin): an antimalarial drug from China. *Science* **228**, 1049-1055.
2. Golenser J, Waknine JH, Krugliak M, Hunt NH, Grau GE (2006) Current perspectives on the mechanism of action of artemisinins. *Int J Parasitol* **36**: 1427-41
3. Krishna S, Bustamante L, Haynes RK, Staines HM (2008) Artemisinins: their growing importance in medicine. *Trends Pharmacol Sci* **29**: 520-7
4. R.K.Haynes, B.Fugmann, J.Stetter, K.Rieckmann, H.D.Heilmann, H.W.Chan, M.K.Cheung, W.L.Lam, H.N.Wong, S.L.Croft, L.Vivas, L.Rattray, L.Stewart, W.Peters, B.L.Robinson, M.D.Edstein, B.Kotecka, D.E.Kyle, B.Beckermann, M.Gerisch, M.Radtke, G.Schmuck, W.Steinke, U.Wollborn, K.Schmeer, A.Romer (2006). Artemisone--a highly active antimalarial drug of the artemisinin class. *Angew.Chem.Int.Ed Engl.* **45**, 2082-2088.
5. G.Q.Li, K.Arnold, X.B.Guo, H.X.Jian, L.C.Fu (1984). Randomised comparative study of mefloquine, qinghaosu, and pyrimethamine-sulfadoxine in patients with falciparum malaria. *Lancet* **2**, 1360-1361.
6. G.Q.Li, X.B.Guo, L.C.Fu, H.X.Jian, X.H.Wang (1994). Clinical trials of artemisinin and its derivatives in the treatment of malaria in China. *Trans.R.Soc.Trop.Med.Hyg.* **88 Suppl 1**, S5-S6.
7. WHO Guidelines for the treatment of malaria (2006).
8. L.Vivas, L.Rattray, L.B.Stewart, B.L.Robinson, B.Fugmann, R.K.Haynes, W.Peters, S.L.Croft (2007). Antimalarial efficacy and drug interactions of the novel semi-synthetic endoperoxide artemisone in vitro and in vivo. *J.Antimicrob.Chemother.* **59**, 658-665.
9. M.Ramharter, D.Burkhardt, J.Nemeth, A.A.Adegnika, P.G.Kremsner (2006). In vitro activity of artemisone compared with artesunate against Plasmodium falciparum. *Am.J.Trop.Med.Hyg.* **75**, 637-639.
10. J.Nagelschmitz, B.Voith, G.Wensing, A.Roemer, B.Fugmann, R.K.Haynes, B.M.Kotecka, K.H.Rieckmann, M.D.Edstein (2008). First assessment in humans of the safety, tolerability, pharmacokinetics, and ex vivo pharmacodynamic

antimalarial activity of the new artemisinin derivative artemisone. *Antimicrob.Agents Chemother.* **52**, 3085-3091.

11. H.J.Woerdenbag, T.A.Moskal, N.Pras, T.M.Malingre, F.S.el-Feraly, H.H.Kampinga, A.W.Konings (1993). Cytotoxicity of artemisinin-related endoperoxides to Ehrlich ascites tumor cells. *J.Nat.Prod.* **56**, 849-856.
12. T.Efferth, H.Dunstan, A.Sauerbrey, H.Miyachi, C.R.Chitambar (2001). The anti-malarial artesunate is also active against cancer. *Int.J.Oncol.* **18**, 767-773.
13. H.H.Chen, H.J.Zhou, X.Fang (2003). Inhibition of human cancer cell line growth and human umbilical vein endothelial cell angiogenesis by artemisinin derivatives in vitro. *Pharmacol.Res.* **48**, 231-236.
14. I.Nakase, H.Lai, N.P.Singh, T.Sasaki (2008). Anticancer properties of artemisinin derivatives and their targeted delivery by transferrin conjugation. *Int.J.Pharm.* **354**, 28-33.
15. L.N.Li, H.D.Zhang, S.J.Yuan, Z.Y.Tian, L.Wang, Z.X.Sun (2007). Artesunate attenuates the growth of human colorectal carcinoma and inhibits hyperactive Wnt/beta-catenin pathway. *Int.J.Cancer* **121**, 1360-1365.
16. H.Chen, B.Sun, S.Pan, H.Jiang, X.Sun (2009). Dihydroartemisinin inhibits growth of pancreatic cancer cells in vitro and in vivo. *Anticancer Drugs* **20**, 131-140.
17. T.G.Berger, D.Dieckmann, T.Efferth, E.S.Schultz, J.O.Funk, A.Baur, G.Schuler (2005). Artesunate in the treatment of metastatic uveal melanoma--first experiences. *Oncol.Rep.* **14**, 1599-1603.
18. N.P.Singh, V.K.Panwar (2006). Case report of a pituitary macroadenoma treated with artemether. *Integr.Cancer Ther.* **5**, 391-394.
19. Z.Y.Zhang, S.Q.Yu, L.Y.Miao, X.Y.Huang, X.P.Zhang, Y.P.Zhu, X.H.Xia, D.Q.Li (2008). [Artesunate combined with vinorelbine plus cisplatin in treatment of advanced non-small cell lung cancer: a randomized controlled trial]. *Zhong.Xi.Yi.Jie.He.Xue.Bao.* **6**, 134-138.
20. Hou, D.Wang, R.Zhang, H.Wang (2008). Experimental therapy of hepatoma with artemisinin and its derivatives: in vitro and in vivo activity, chemosensitization, and mechanisms of action. *Clin.Cancer Res.* **14**, 5519-5530.
21. N.P.Singh, H.C.Lai (2004). Artemisinin induces apoptosis in human cancer cells. *Anticancer Res.* **24**, 2277-2280.

22. H.J.Zhou, Z.Wang, A.Li (2008). Dihydroartemisinin induces apoptosis in human leukemia cells HL60 via downregulation of transferrin receptor expression. *Anticancer Drugs* **19**, 247-255.
23. H.H.Chen, H.J.Zhou, W.Q.Wang, G.D.Wu (2004). Antimalarial dihydroartemisinin also inhibits angiogenesis. *Cancer Chemother.Pharmacol.* **53**, 423-432.
24. M.Wartenberg, S.Wolf, P.Budde, F.Grunheck, H.Acker, J.Hescheler, G.Wartenberg, H.Sauer (2003). The antimalaria agent artemisinin exerts antiangiogenic effects in mouse embryonic stem cell-derived embryoid bodies. *Lab Invest* **83**, 1647-1655.
25. E.Buommino, A.Baroni, N.Canozo, M.Petrazzuolo, R.Nicoletti, A.Vozza, M.A.Tufano (2009). Artemisinin reduces human melanoma cell migration by down-regulating alpha V beta 3 integrin and reducing metalloproteinase 2 production. *Invest New Drugs* **27**, 412-418.
26. I.R.Ribeiro, P.Olliaro (1998). Safety of artemisinin and its derivatives. A review of published and unpublished clinical trials. *Med.Trop.(Mars.)* **58**, 50-53.
27. T.Gordi, E.I.Lepist (2004). Artemisinin derivatives: toxic for laboratory animals, safe for humans? *Toxicol.Lett.* **147**, 99-107.
28. Adjuik M, Babiker A, Garner P, Olliaro P, Taylor W, White N; International Artemisinin Study Group. Artesunate combinations for treatment of malaria: meta-analysis. Lancet. 2004 Jan 3;363(9402):9-17.
29. Liu WM. Enhancing the cytotoxic activity of novel targeted therapies--is there a role for a combinatorial approach? Curr Clin Pharmacol. 2008 May;3(2):108-17. Review.
30. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970 Aug 15;227(5259):680-5.
31. Liu WM, Scott KA, Shamash J, Joel S, Powles TB. Enhancing the in vitro cytotoxic activity of Delta9-tetrahydrocannabinol in leukemic cells through a combinatorial approach. Leuk Lymphoma. 2008 Sep;49(9):1800-9.
32. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul. 1984;22:27-55.
33. K.T.Batty, T.M.Davis, L.T.Thu, T.Q.Binh, T.K.Anh, K.F.Ilett (1996). Selective high-performance liquid chromatographic determination of artesunate and alpha-

and beta-dihydroartemisinin in patients with falciparum malaria. *J.Chromatogr.B Biomed.Appl.* **677**, 345-350.

34. T.Efferth, A.Sauerbrey, A.Olbrich, E.Gebhart, P.Rauch, H.O.Weber, J.G.Hengstler, M.E.Halatsch, M.Volm, K.D.Tew, D.D.Ross, J.O.Funk (2003). Molecular modes of action of artesunate in tumor cell lines. *Mol.Pharmacol.* **64**, 382-394.
35. A.C.Beekman, A.R.Barentsen, H.J.Woerdenbag, U.W.Van, N.Pras, A.W.Konings, F.S.el-Feraly, A.M.Galal, H.V.Wikstrom (1997). Stereochemistry-dependent cytotoxicity of some artemisinin derivatives. *J.Nat.Prod.* **60**, 325-330.
36. Y.Li, F.Shan, J.M.Wu, G.S.Wu, J.Ding, D.Xiao, W.Y.Yang, G.Atassi, S.Leonce, D.H.Caignard, P.Renard (2001). Novel antitumor artemisinin derivatives targeting G1 phase of the cell cycle. *Bioorg.Med.Chem.Lett.* **11**, 5-8.
37. J.A.Willoughby, Sr., S.N.Sundar, M.Cheung, A.S.Tin, J.Modiano, G.L.Firestone (2009). Artemisinin blocks prostate cancer growth and cell cycle progression by disrupting Sp1 interactions with the cyclin-dependent kinase-4 (CDK4) promoter and inhibiting CDK4 gene expression. *J.Biol.Chem.* **284**, 2203-2213.
38. Wu J, Feng Y, Xie D, Li X, Xiao W, Tao D, Qin J, Hu J, Gardner K, Judge SI, Li QQ, Gong J. (2006) Unscheduled CDK1 activity in G1 phase of the cell cycle triggers apoptosis in X-irradiated lymphocytic leukemia cells. *Cell Mol Life Sci* **63**(21), 2538-2545
39. O'Neill PM, Barton VE, Ward SA. (2010) The molecular mechanism of action of artemisinin--the debate continues. *Molecules*. **15** (3):1705-21.
40. N.P.Singh, H.C.Lai (2005). Synergistic cytotoxicity of artemisinin and sodium butyrate on human cancer cells. *Anticancer Res.* **25**, 4325-4331.
41. T.Chen, M.Li, R.Zhang, H.Wang (2009). Dihydroartemisinin induces apoptosis and sensitizes human ovarian cancer cells to carboplatin therapy. *J.Cell Mol.Med.* **13**, 1358-1370.
42. H.J.Zhou, J.L.Zhang, A.Li, Z.Wang, X.E.Lou (2009). Dihydroartemisinin improves the efficiency of chemotherapeutics in lung carcinomas in vivo and inhibits murine Lewis lung carcinoma cell line growth in vitro. *Cancer Chemother.Pharmacol.*
43. T.Hata, H.Yamamoto, C.Y.Ngan, M.Koi, A.Takagi, B.Damdinsuren, M.Yasui, Y.Fujie, T.Matsuzaki, H.Hemmi, X.Xu, K.Kitani, Y.Seki, I.Takemasa, M.Ikeda,

M.Sekimoto, N.Matsuura, M.Monden (2005). Role of p21waf1/cip1 in effects of oxaliplatin in colorectal cancer cells. *Mol.Cancer Ther.* **4**, 1585-1594.

44. C.Tolis, G.J.Peters, C.G.Ferreira, H.M.Pinedo, G.Giaccone (1999). Cell cycle disturbances and apoptosis induced by topotecan and gemcitabine on human lung cancer cell lines. *Eur.J.Cancer* **35**, 796-807.
45. L.Escoubet-Lozach, I.L.Lin, K.Jensen-Pergakes, H.A.Brady, A.K.Gandhi, P.H.Schafer, G.W.Muller, P.J.Worland, K.W.Chan, D.Verhelle (2009). Pomalidomide and lenalidomide induce p21 WAF-1 expression in both lymphoma and multiple myeloma through a LSD1-mediated epigenetic mechanism. *Cancer Res.* **69**, 7347-7356.
46. C.Riganti, S.Doublier, D.Viarisio, E.Miraglia, G.Pescarmona, D.Ghigo, A.Bosia (2009). Artemisinin induces doxorubicin resistance in human colon cancer cells via calcium-dependent activation of HIF-1alpha and P-glycoprotein overexpression. *Br.J.Pharmacol.* **156**, 1054-1066.

Table I

IC50 (μM)	Artemisinin	Artemisone
Colon		
HCT116	204 ± 88	9.5 ± 0.9
SW480	156 ± 38	4.5 ± 1.6
Melanoma		
MJT3	>1,000	30 ± 26
KM	101 ± 15	6.4 ± 2.1
Pancreas		
PANC1	>1,000	>1,000
MiaPaCa3	31 ± 13	14 ± 10
Breast		
MCF7	44 ± 15	0.56 ± 0.17

Legends to Tables and Figures

Table 1 Comparison of IC50 values obtained for ART and ATM in seven cell lines. IC50 values were taken from the cell viability curves and represent the concentration needed to reduce the cell population by half after 72 hours treatment with the drug. The IC50s for ATM were consistently lower than that of ART in all cell lines. Each value represents the mean and SDs of at least 3 separate experiments.

Figure 1 Cell viability curves in response to ART and ATM as measured by MTT assay for seven cancer cell lines. Cells were exposed to a range of drug concentrations for 72hrs, before measuring the number of viable cells by absorbance at 550nm. ATM decreased cell number to a greater degree than ART when used at equi-active concentrations. Data points represent the mean and SDs of at least 3 separate experiments. Data points associated with * for ART or # for ATM indicate the lowest concentration for which the difference between untreated cell number and treated cell number is significant ($p<0.01$) for that drug in that cell line. All subsequent data points (higher concentrations) are also significant.

Figure 2 PI flow cytometric histograms showing the proportion of cells at sub-G1(apoptotic (A)), G1, S and G2 phases of the cell cycle in response to equi-active concentrations of the ART and ATM in three different cell lines. Graph shows untreated versus treated for three separate experiments. MCF7 cells treated with ATM exhibited a significantly greater proportion of cells in G1. Conversely, significantly smaller percentages of cells within G1 were seen in SW480 cells. *$p<0.05$, **$p=0.015$

Figure 3 a) Effects of ART and ATM on levels of intracellular cycling proteins as viewed by western blot. Cells were untreated or treated with equi-active amounts of the two drugs for 72hrs before running total cellular protein and probing with appropriate antibody. Representative blots are shown. b) Protein bands were quantified using densitometry techniques, normalised to GAPDH loading controls and then percentage change in protein level compared to untreated controls was calculated. Graphs show mean protein change and SDs of 3 separate experiments

Figure 4 Combinations were analysed using median effect analysis (**a**) or modulating dose model (**b & c**). **a.** Combining OXP with the artemisinins produces differing effects depending on the derivative used. The median effect equation was used to generate CI values which, when above 1 indicate antagonism and below 1 enhancement of effect. **b.** Small doses of GEM were used to modulate the IC50 of ART and ATM in different cell lines. IC50 is greatly increased when GEM is added to ART whilst this effect is less prevalent with ATM. **c.** Combination with THAL did not greatly affect the IC50 of ART or ATM in any of the cell lines. All values represent a mean and SDs of at least 3 experiments