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The antimalarial agent artesunate possesses anticancer properties that can be enhanced by combination strategies

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Artemisinins are a class of compounds that are first-line treatment options for malaria. They also have potent antiproliferative activity, which makes them potential anticancer drugs. We have previously demonstrated anticancer activity of a number of these compounds *in vitro*; however, cytotoxic activities were compromised by drug-induced cell cycle arrests. In this study, we have explored further the activity of the clinical lead artemisinin-drug artesunate (ART), used either alone or in combination with established chemotherapy. Also, by using a cell line expressing polyploidy character, have also explored the impact of cell cycle arrest in determining overall drug activity. Results showed that ART caused dose-dependent decreases in cell number, which were associated with either increased cytotoxicity or cytostasis. Cytostasis appeared to be a consequence of a simultaneous arrest at all phases of the cell cycle, a deduction that was supported by molecular profiling, which showed reductions in cell cycle transit proteins. ART appeared to maintain cells in this arrested state; however, reculturing these treated cells in drug-free medium resulted in significant reductions in viabilities. We also showed that ART maintained activity in polyploidy cells, and that an impressive enhancement to its activity was achievable through a combination with the immunomodulatory drug lenalidomide. Taken together, these observations indicate ART and its related compounds may be effective for the treatment of tumours, and that activity is related to schedule. Therefore, it is important to carefully select the most appropriate schedule to maximise ART efficacy.

Herbal remedies involving the Sweet wormwood (*Artemisia annua*) have been traditionally used in China as a treatment for a variety of ailments, including fever and rheumatism. The major active ingredient artemisinin was identified and isolated in the 1970s, and its particular activity as a compound targeting malaria investigated ever since. Structural activity relationship programmes have refined the parental artemisinin down three main avenues. One of these have focussed on developing the hemisuccinate ester of artemisinin known as artesunate (ART), which taken together with its siblings have resulted in a family of compounds collectively known as the "artemisinins." These agents are amongst the most potent and rapidly acting antimalarial agents known,[1] and are efficacious against parasites that are resistant to established antimalarial drugs such as chloroquine and pyrimethamine.[2] The mechanism of action of artemisinins has yet to be defined, but studies have described a role for free radicals. Specifically, induced de novo production of reactive hydroxyl moieties and superoxides within the malarial parasite have been reported that damages intracellular processes and causes death.[3,4] In spite of the lack of a definitive mechanism, the World Health Organisation have recommended that all antimalarial therapies should contain an artemisinin component, especially when used as a first-line treatment.[5]

There is growing evidence supporting a role of ART and other artemisinins in cancer therapy.[6–13] Antiproliferative activity has been described *in vitro* for this class of agent in a wide spectrum of tumour cell lines as well as in primary material derived from patients. The mechanism(s) underlying these activities are unclear, and have included (*i*) actions on cell cycle proteins that determine transit through G1 restriction,[14] (*ii*) disruptions to the intrinsic apoptotic pathway that drive it toward a proapoptotic outcome,[11,13,15] (*iii*) antiangiogenic and antimetastatic properties[16–18] and (*iv*) inhibition of NF-kB.[19,20] The diversity in the targets of ART, naturally lends support to the possibility that it be used in combination with other agents that mutually support each other.[21] Indeed, we and others have reported nonantagonistic interactions between ART and ART-related compound with common anticancer drugs,[12,22] as well as enhanced activities in combinations with more novel treatment modalities such as erlotinib and rituximab.[23,24]

There is currently limited published data exploring the value of ART as a combination partner in treatment regimens. These studies have used simple approaches to studying drug–drug interactions, and as a consequence, their conclusions are still open to debate. Therefore, the aim of this study was first, to assess the antiproliferative effect of ART in a panel of

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cancer cell lines *in vitro* and second, to investigate for possible interactions between ART and established chemotherapy agents by using an array of more detailed methodologies. This would allow us to test the general concept that drugs with similar mechanistic profiles to ART could be used in combination to enhance the overall susceptibility of cancer cells to these drugs.

Material and Methods

Drugs

Artesunate (ART: Pharmacy, St George's Hospital (SGH), UK) and lenalidomide (LEN: Celgene Corp., Summit, NJ, USA) were both dissolved in dimethyl sulphoxide, and gemcitabine (GEM: SGH) and oxaliplatin (OXP: Sigma Ltd., Poole, Dorset, UK) in phosphate buffered saline (PBS). All drugs were reconstituted to a concentration of 10 mM and stored at −20°C for no longer than 4 weeks.

Cell culture

The human cancer cell lines, MCF7 (breast), HCT116 (colon) and A549 (lung) (Cancer Research UK, London, UK), were grown in either DMEM (Sigma) or RPMI (Sigma) media supplemented with 10% foetal bovine serum, 2 mM L-glutamine and 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.

A HCT116 cell line variant that possessed a large population of cells that exhibited a ploidy greater than 4n was developed by culturing HCT116 cells in standard growth medium for 3 months and at passages >30. This extended-passage cell line, was designated $HCT116^{poly}$, had a fraction of cells with a higher DNA content, which displayed growth characteristics and doubling times that were similar to the parent HCT116 ($HCT116^{norm}$).

Proliferation assays

To study the effect of each agent on cell growth, cells growing exponentially were added to 96-well plates at a density of 3×10^4 /well. Drugs (0.1–100 μM) were then added to the wells, ensuring an equal volume of 200 μl across the plate. Cell number was measured at 72 hr using a standard methylthiazoletetrazolium (MTT)-based assay without modifications as described previously.[25] Briefly, MTT (Sigma) was added to each well to give a working concentration of 0.4 mg/ml, and plates returned to the incubator for a further 1 hr. After this time, the medium was aspirated off, 200 μl 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 ART (1–100 μM) for 72 hr, before fixing in 70% (v/v) ethanol in PBS. Following an incubation period of at least 30 min, cells were washed and resuspended in a DNA staining solution (1 mg/ml propidium iodide and 1 ng/ml RNAse A (both Sigma). Acquisition of data was performed within 1 hr using a Becton Dickinson FACSCalibur (BD Biosciences), and gating on fluorescence width and area was used 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. Following transfer of proteins to 0.45-μm nitrocellulose membranes, blocking was performed in 5% (w/v) nonfat milk in TTBS [0.5% (v/v) Tween-20 in TBS (50 mM Tris, 150 mM NaCl, pH 8.0)]. Primary antibody probing was performed with anti-p21, anti-Bax, anti-CDK4, anti-cyclin D1, anti-pRb or anti-cyclin B1. All primary antibodies were obtained from New England Biolabs (Hitchin, UK) and used at a dilution of 1:1,000, unless stated otherwise. Anti-GAPDH was used as a loading control (1:2,000—New England). Following three 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 by the ECL-plus detection system (Amersham).

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

Our combination studies followed an approach previously described.[25] $HCT116^{norm}$, A549, MCF7 and $HCT116^{poly}$ cells (5×10^4 /well) growing exponentially were reset in fresh culture medium and aliquoted into 96-well plates. ART was the primary drug partner and combined with OXP at an equal ratio of their respective IC50 (*e.g.*, $1/2\times$ IC50 of ART was combined with $1/2\times$ IC50 of OXP). Fixing this ratio also kept constant the amount of one drug respective to the other. For instance, ART was combined with OXP at a constant ratio of 1:2.5. 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 the natures of drug–drug interactions then assessed by calculating a combination index (CI) by using the median-effect equation, 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 GEM or LEN (modulating agent) and testing its ability to influence the activity of ART (drug partner). Cells

(5×10^4 /well) growing exponentially were reset in fresh culture medium and aliquoted into 96-well plates. ART was 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 coculture with a suboptimal concentration (~IC20) of GEM or LEN 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 ART in the presence and absence of the combinatorial drug partner.

Recovery studies

The effect of recovery from the drug was studied by studying the impact that removing drugs from cells would have on cell growth and survival. A549, MCF7 and HCT116^{poly} cells growing exponentially were reset at 5×10^4 /well and allowed to establish for 24 hr, before adding 30 μM ART. Following 2-days culture, media were aspirated, and the cells washed twice with PBS. Fresh culture medium was then returned to the cells with or without ART (30 μM) and incubated 2 days further. Cell number and viability were then assessed on Days 2 and 4; with percentages of live and dead cells discriminated by trypan blue dye exclusion.

Results

ART reduces cell number by being both cytotoxic and/or cytostatic

Three commonly used anticancer agents were selected for investigation on the basis of their activities in the cancer types studied, as well as on preliminary studies that showed similar modes of action. There were dose-dependant reductions in cell numbers as established by MTT scores in all cell lines cultured with ART, CPM, LEN and GEM, with IC50 values showing that A549 cells were generally less sensitive to the cytotoxic effects of the four drugs (Table 1). The suboptimal concentrations used in the combination experiments were decided by more detailed analyses of emax-modelling of data around the top of the dose-response curve, and were those that caused no more than 10% cell death.

The reductions in cell numbers caused by ART were recapitulated by cell counting using microscopy and viability discrimination by trypan blue exclusion (Fig. 1*a*). This also revealed that the reductions in cells numbers in HCT116 cells seen after culturing with ART was associated with a dose-dependent reduction in cell viability. However, there were no decreases in the cell viability of A549 and MCF7 cells cultured with ART, which suggested a cytostatic effect (Fig. 1*a*).

Flow cytometric analyses revealed culturing cells with ART induced clear dose-dependent increases in the sub-G1 (apoptotic/debris) population in HCT116 cells but no changes in A549 and MCF7 cells (Fig. 1*b*). There were no dramatic changes to any of the cell cycle phases in A549 cells, which supported the overall notion of a simultaneous blockade in cell progression at all phases of the cell cycle. A similar broad-spectrum cell cycle arrest was also seen in MCF7 cells cultured with higher concentrations of ART (>30 μM). However, flow cytometry showed significant increases in the percentage of cells within G1 and concomitant decreases in cell within the S-phase of the cell cycle, which pointed to more specific blockades at the G1-to-S and G2-to-M transitional points with concentrations <30 μM.

Table 1. IC50-values (μM) at 72 hr for ART, LEN, GEM and OXP in A549, HCT116^{norm} and MCF7 cell lines. The values were established by the MTT assay, and represent the means and SDs of at least five independent experiments

	Cell line		
IC50 (μM)	A549	HCT116^{norm}	MCF7
Artesunate	9.8 ± 1.7	1.92 ± 0.35	2.3 ± 0.94
Lenalidomide	>100	>100	>100
Gemcitabine	14 ± 1.6	5.2 ± 2.02	1.9 ± 0.75
Oxaliplatin	6.2 ± 0.95	4.05 ± 1.8	6.1 ± 1.1

ART alter proteins that regulate G1 transit

To investigate the effect of ART on cell proliferation, whole cell lysates obtained from cell lines cultured with ART were immunoprobed for CDK4 and cyclin D—the first-line protagonists that initiate and drive cells through the restriction point. Both proteins, upon treatment with ART, were reduced in a dose-dependent manner (Fig. 2). There were also concomitant reductions in pRb at similar concentrations, which was clearest in A549 and MCF7 cells. The level of expression of p21$^{waf1/cip1}$ was dramatically increased in HCT116 and MCF7 cells but unchanged in A549 cells. This was most clearly demonstrated in the densitometry graphs (Fig. 2).

ART acts additively with chemotherapy to reduce cell number and viability

We used MTT assays to establish drug-combination activity, and calculated CI-values to determine the nature of drug–drug interactions. These computations require the tested drugs to respond in a dose-dependent effect, which permits the calculation/extrapolation of IC50-values that are shown in Table 1. However, these values could not be generated for LEN and were not used this model. CI-values were generated from data that were generally no different from 1, which suggested an additive interaction of ART with GEM or with OXP (Fig. 3*a*). There was the faintest hints of antagonism with ART and GEM combinations in HCT116, but this did not reach significance ($p = 0.073$).

The cytotoxic effects of ART is enhanced by LEN

The lack of a dose-response in our cells rendered our primary model of drug–drug interaction ineffectual for combinations of ART with LEN. For this reason, we next tested the ability of LEN to sensitise cells to ART. These combinatorial analyses were performed in all the cell lines using a smaller concentration of LEN (0.1 and 1 μM). These concentrations

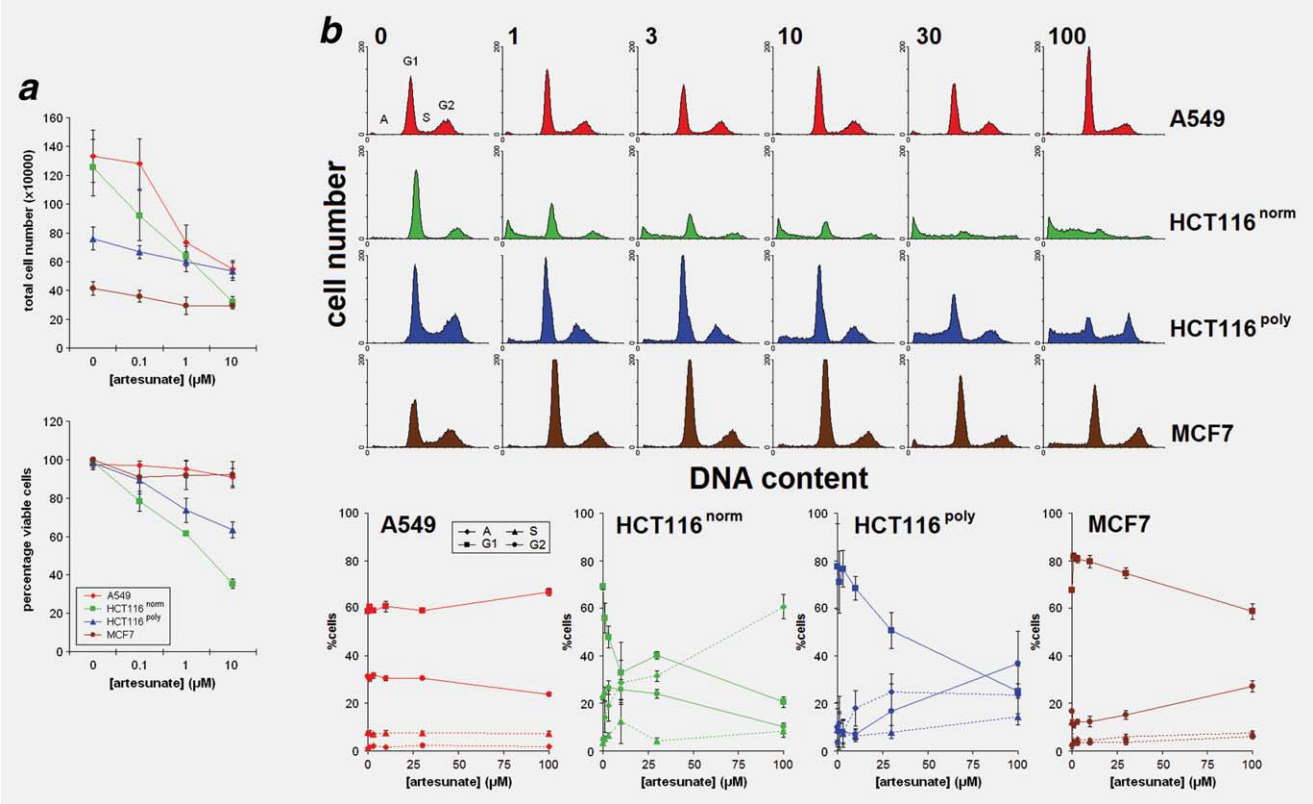


Figure 1. Effect of ART on cell proliferation and cell cycle dynamics. A549, HCT116^{norm}, HCT116^{poly} and MCF7 cells were cultured with ART (0–100 μM) for 72 hr before assessing cell number and viability by cell counting with trypan blue dye discrimination (*a*). Each data point is the mean and SDs of three separate experiments. Cell cycle distribution in sub-G1 (A), G1, S and G2 by propidium iodide staining and flow cytometric analysis of cells are shown in (*b*). There were significant increases in the sub-G1 population in HCT116^{norm} and HCT116^{poly} cells that indicated cytoxicity. Conversely, no clear changes to profiles for A549 and MCF7 cells suggested a general global cell cycle arrest. Representative histograms are represented in the upper panel, and the means and SDs of at least four independent replicates are represented in the lower line graphs. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

had little effect on cell numbers and viabilities. Results showed that this subtoxic amount of LEN had no significant impact on the response of HCT116^{norm} cells to ART. However, these modulatory dose of LEN significantly enhanced the effect of ART in A549 and MCF7 cells as indicated by a fall in their IC50-values (Fig. 3*b*).

Generation of ART-resistant polyploid HCT116 cells

HCT116 cells that were passaged and maintained using standard cell culture procedures for ~3 months spontaneously developed a polyploidy phenotype. These cells, designated HCT116^{poly} exhibited similar growth characteristics, with a population-doubling time of about 28 h (27.6 ± 5.9 for HCT116^{poly} *vs.* 28.2 ± 3.9 for HCT116^{norm}). The percentage of HCT116^{poly} cells located within the polyploid continuum (where ploidy was >2) was increased by nearly threefold (39 ± 10% *vs.* 14 ± 1.4%; $p < 0.001$), with clear definitions of cells in 8n, 16n and 32n (Figs. 4*a* and 4*b*).

The sensitivity of HCT116^{poly} cells to ART was noticeably reduced, with an IC50-value of 39 ± 2.6 μM, which was significantly higher than that seen in the parental HCT116^{norm} cell (1.9 ± 0.35 μM; $p < 0.001$) (Figs. 1*a*, 4*c* and 4*d*). Flow cytometric analyses of cells following culturing with ART revealed a dose dependent increase in the sub-G1 population of cells, which was associated with a reduction in the polyploid fraction, a reduction in the %G1 events and an increase in the %G2 cells (Figs. 1*b*, 4*c* and 4*d*). The molecular profile of HCT116^{poly} cells cultured with ART was generally the same as that of the HCT116^{norm} (Fig. 2). However, differences were seen with cyclin B (reduced in HCT116^{norm} but unchanged in HCT116^{poly}) and BAX (unchanged in HCT116^{norm} but decreased in HCT116^{poly}) (Figs. 4*c* and 4*d*).

ART cytotoxicity in HCT116^{poly} cells is enhanced by a recovery phase

To investigate whether the G2-block observed in HCT116^{poly} was preventing cell death[26] and was inadvertently maintained in the presence of ART, these cells were cultured in 30 μM ART for 2 days before removal of the drug and recovery in drug-free medium for a further 2 days. Results showed reculturing ART-treated HCT116^{poly} cells in drug-free medium caused significant decreases in cell viability (Fig. 5*a*).

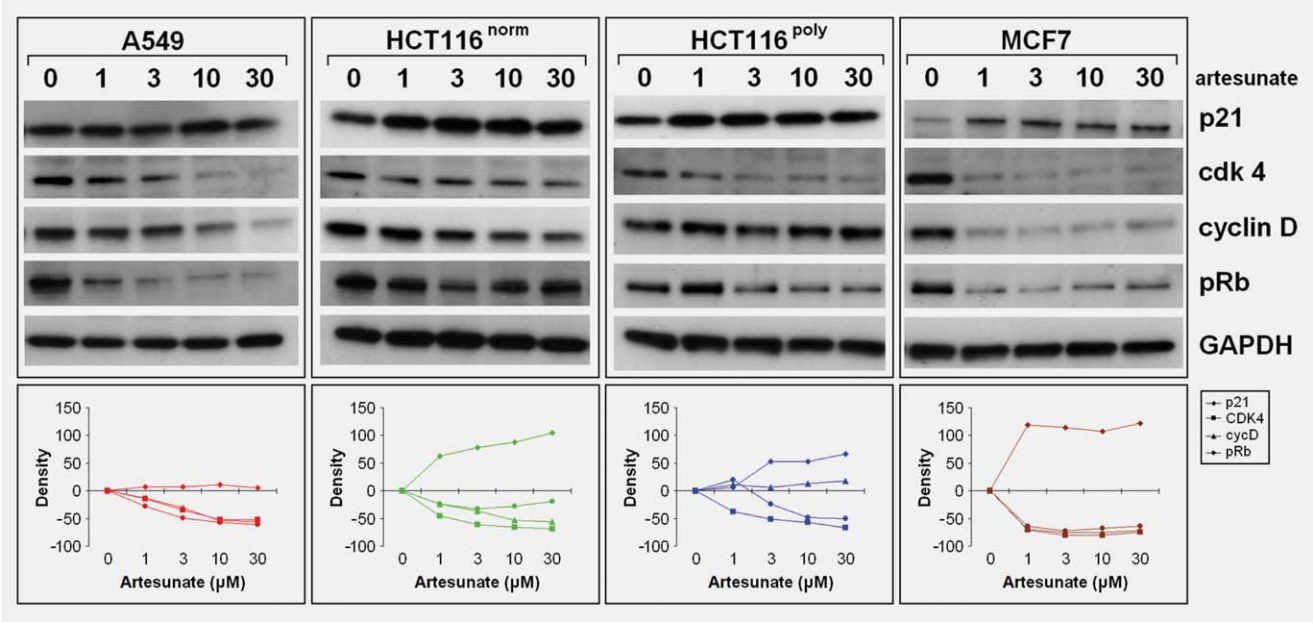


Figure 2. Effect of ART on proteins that regulate transit through the G1 restriction point. Each of the cell lines were cultured with ART (0–30 μM) for 72 hr before western blotting for the proteins indicated. There were decreased expressions of pRb in all the cell lines, which were associated with reductions in CDK4 and/or cyclinD. These were generally mirrored by increases in p21. Representative blots are shown, and data from densitometric analysis of three independent experiments are shown in the lower line graphs. SDs have been omitted for clarity, but coefficient of variances for most points were <10%. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

This was associated with an easing of the G2-block, and concomitant increases in the sub-G1 (apoptotic/dead) cell population (Fig. 5*a*).

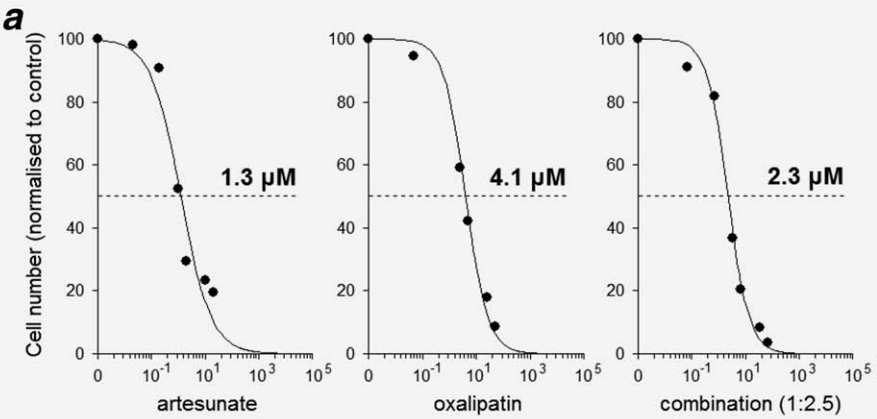
Similar results were seen in both A549 and MCF7 cells following an equivalent treatment-recovery schedule, with significant losses in cell viabilities after reculturing ART treated cells in drug-free medium, compared to cells cultured in 30 μM ART continuously for 4 days ($p < 0.001$) (Fig. 5*b*).

Discussion

These studies were undertaken as part of our larger remit to investigate whether or not immunotherapies could enhance the activities of other modalities and, thus, improve the outcome and quality of life in cancer patients. An avenue of research has involved exploratory studies with drugs that possess anticancer properties but are not typically used in an oncological setting. These drugs have established therapeutic roles in their respective indications and are, therefore, safe to use; however, their potential in other disease types may not have been explored thoroughly. In this study, we specifically investigated the antiproliferative effects of the malarial compound ART on a panel of cancer cell lines. By using *in vitro* models, we assessed its effects on cell growth and survival, and established the value of drug-combination strategies that included its use. In summary, our results showed ART possessed good antitumour effects, which were associated with modifications to cell cycle functionality. Furthermore, efficacy could be enhanced by combining ART with other drugs, and by adapting treatment schedules to include drug-free periods.

Recent research activities have focussed on the anticancer potential of the artemisinin-class of antimalarial agents. These drugs have potent cytotoxic activity against the malaria-causing *Plasmodium sp.* and are regarded as a first line therapeutic drug to be used in combination with other antimalarial modalities.[5] They have also been shown to have anticancer value by dint of their cytotoxic mechanism. These drugs, as exemplified by ART, have reported anticancer activity *in vivo* and have been used successfully in patients with metastatic melanoma.[22] Currently, there are a number of phase I trials assessing activity and tolerability of ART in patients with cancers of the colon* and breast†, which endorses the validity of this drug as a therapeutic candidate. Although a definitive mechanism responsible for cell killing has yet to be completely defined, early studies showed ART to be rapidly converted intracellularly into reactive oxygen species that could disrupt cellular function.[27] Research has rapidly progressed in recent years, and a multitude of mechanisms have been suggested. Currently, activity has been associated with interference of principal signalling pathways,[28] antimetastasis,[18] DNA damage[29] and cell cycle modifications.[14] Indeed, we recently reported that the antiproliferative properties of two ART-related compounds were associated with potent cell cycle

*International standard randomised controlled trial number: ISRCTN05203252. http://www.controlled-trials.com/ISRCTN05203252.3
†ClinicalTrials.gov Identifier: NCT00764036. http://clinicaltrials.gov/ct2/show/record/NCT00764036.



$$CI = \left[\frac{(D)_1}{(D_{50})_1} + \frac{(D)_2}{(D_{50})_2}\right] = 0.82$$

	Cell line		
	A549	HCT116^{norm}	MCF7
+GEM	1.2 ± 0.27	1.4 ± 0.32	0.96 ± 0.17
+OXP	0.87 ± 0.094	0.95 ± 0.31	0.99 ± 0.22

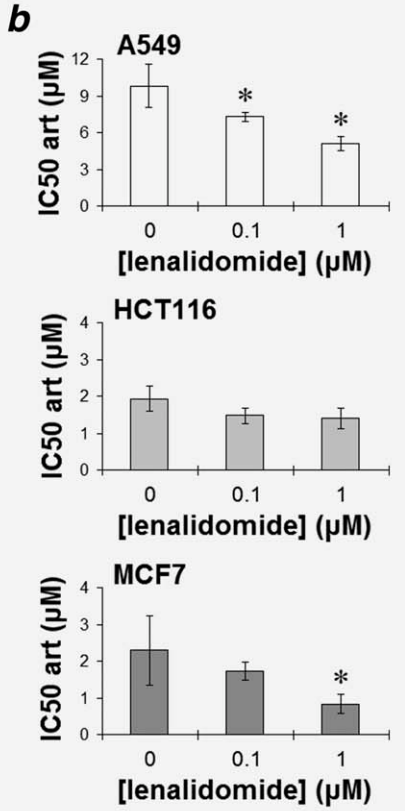


Figure 3. Effect of combining ART with chemotherapy in A549, HCT116^{norm} and MCF7 cells. Cells were cultured concomitantly with ART and GEM or ART and OXP for 72 hr, before assessing cell numbers by MTT (*a*). Nonexclusive CI values for the 50% unaffected fraction were calculated and shown in the lower panel. CI-values >1 indicates antagonis; CI = 1 additivity and CI < 1 synergy. Representative response curves and the extrapolated IC50-values for ART, OXP and a combination of the two drugs (combined at equi-molar fractions of respective IC50 values, which was at a ratio of 1:2.5) are shown in the upper panel. Cells were also cultured with a range of ART in the absence or presence of small nongrowth inhibitory concentrations of LEN (0.1 and 1 μM), to assess the effects of these doses on the responses of the cells to ART (*b*). There were significant enhancements of ART activity as indicated by reductions in the IC50s for ART when used in the presence of LEN in A549 and MCF7 cells. Each point is the mean and SDs of at least three independent experiments, and the asterices indicate significant difference compared to the LEN-free culture ($p < 0.001$).

perturbations,[12] which supported the overall notion that these agents may have a role in cancer therapies.

In the first part of this study, we assessed the activity of ART in a small panel of cancer-derived cell lines, and showed through MTT assays, a dose-dependent reduction in cell numbers. The primary aim of this study was to explore the therapeutic potential of ART in a cancer setting both as a single agent and in combination with more common chemotherapy. Consequently, the cell lines were selected on the basis of their being representative of typical cancers. A reduction in cell number can be a consequence of active cell kill or inhibition of proliferation. Consequently, we performed cell counting using light microscopy supported by trypan blue exclusion to discriminate viable from non-viable cells. A reduction in cell viability was seen only in HCT116^{norm} cells, which suggested ART was cytotoxic in this cell line. Conversely, no changes to the viability of A549 and MCF7 but significant reduction in the number of cells as determined by cell counting, suggested a cytostatic response to ART. This deduction was further supported by flow cytometric analysis of cell cycle distribution that revealed ART caused no significant changes to the cell cycle, which suggested a simultaneous arrest at all phases of the cell cycle. This effect has been observed with other derivatives of artemisinin,[14] and associated with downregulation of cyclins and CDKs. Our results recapitulated these earlier results showing clear and significant increases in p21$^{waf1/cip1}$ and concomitant decreases in cyclin D1 and/or CDK4. These proteins are central regulators of transition through G1-initiation, and their reduced levels coupled with loss of pRb, indicated disruption to G1 and S transit. G2-cycling was also affected as indicated by a reduction in cyclin B1. Taken together, ART was a potent cell cycle inhibitor.

The actions and activities of drugs that target cell cycling are interlinked with the ploidy status in the effector cell.[30] In fact, uncoupling DNA synthesis from cytokinesis can give rise to endo-replication and then polyploidy—a phenotype that is

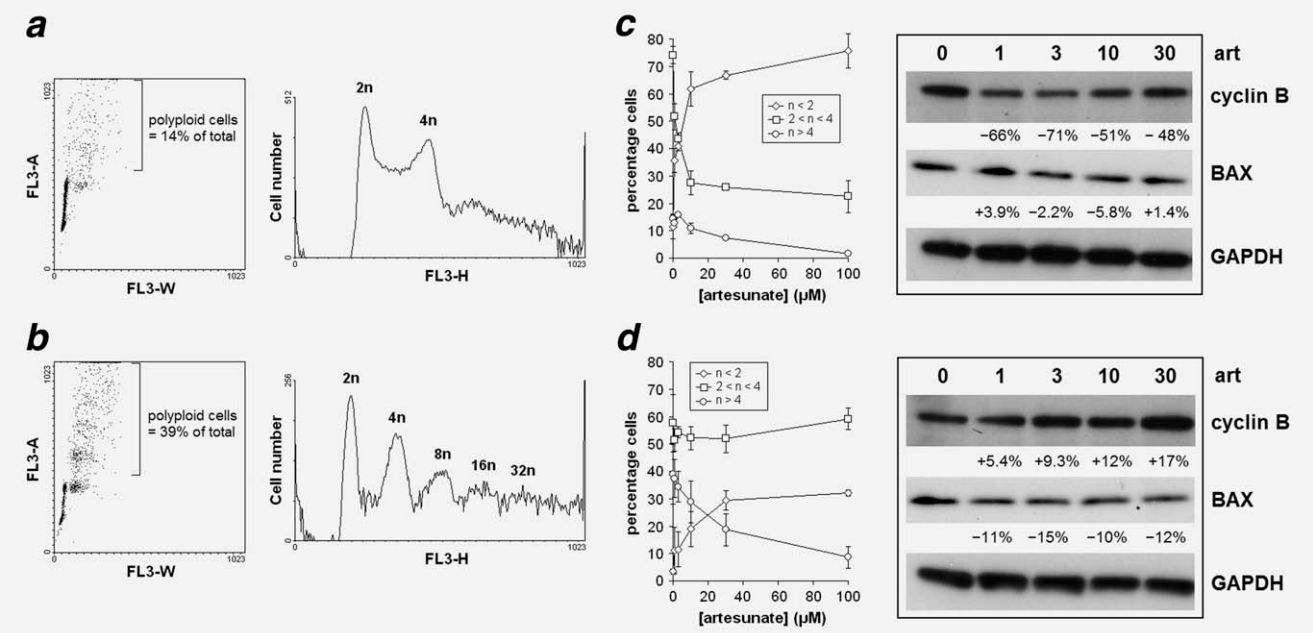


Figure 4. Effect of ART on HCT116^{poly} cells. HCT116^{norm} cells were over-passaged for 10–12 weeks, and the ploidy of these cells (HCT116^{poly}) assessed by using propidium iodide staining and flow cytometry. The percentage of hyperploid cells was increased over the parental HCT116^{norm}, as indicated by increases in the number of cells with a high FL3-A expression but similar FL3-W values (*a, b*). Additionally, distinct hyperploidy populations were observed in the FL3-H channel (*a, b*). Culturing with ART resulted in a G2-blockade and a modest increase in the sub-G1 ($n < 2$) in HCT116^{poly} cells (*c*). This was in contrast to the response in HCT116^{norm} cells, where there was no blockade rather a more pronounced increase in the dying population (*d*). Western blotting data revealed differences in cyclin B and BAX proteins in response to ART. ART was associated with a decrease in cyclin B in HCT116^{norm} cells, but an increase in HCT116^{poly} cells. Similarly, having seen no effect on BAX in HCT116^{norm}, ART caused a decrease in BAX expression in HC116^{poly}. Each data point is the mean and SD of at least three separate experiments. Autoradiograms and representative of three independent experiments, and densitometry results are the mean data for each band normalised to its respective GAPDH loading control. Error bars have been omitted for space.

common in cancer cells.[31] Synchronisation of cell cycle events is achieved by cyclins and their partner CDKs; and disrupting these regulatory proteins, particularly CDK1/cyclin B can lead to polyploidy. Cell cycle inhibitors/modifiers work through these same proteins, and consequently, activity may be influenced by the condition of the ploidy. For this reason, we induced polyploidy in HCT116^{norm} cells and assessed the activity of ART in this variant. Methodologically, polyploidy was induced in the HCT116^{norm} cell line, which is a microsatellite unstable line with MLH1 deficiencies,[32–34] by extending the duration of its maintenance to >3 months. Repeated passage of cells can increase the frequency of polyploidy in cells as a result of increased stress,[35,36] and our method consistently resulted in cells with polyploidy features. These HCT116^{norm} cell variants were designated HCT116^{poly}, and hyperploidy was indicated by a distinct cell continuum above the G1/S/G2/M group of cells specified by FL3-A *vs.* FL3-W analysis. This was confirmed by Giemsa-banding (data not shown), which reported the presence of cells with chromosome numbers >45 (normal range: 43–45).[33]

Results indicated that ART continued to reduce cell numbers in HCT116^{poly} cells. However, the nature of this reduction had now switched to a cytostatic one; with the magnitude of cell death (indicated by %viable cells) being significantly reduced. Flow cytometry showed that the polyploidy fraction of cells was reduced by culturing with ART, which was mirrored by a small increase in the sub-G1 population. However, what was striking was that cells within the normal ploidy-range were now blocking in G2. Furthermore, western blotting showed cyclin B levels, which were down regulated in HCT116^{norm} cells, were now unchanged after treatment in HCT116^{poly}. Taken together, these results suggested that alterations to HCT116^{norm} cells that rendered them polyploidy also made them less sensitive to ART. This resistance, however, was not observed in the polyploidy fraction of cells but only in cells with a normal ploidy. This is reassuring considering a large proportion of cancers are associated with a level of hyperploidy.[31] Resistance in this fraction was associated with a G2-blockade, and appeared to be specific to ART, as the sensitivities to GEM and OXP were similar in HCT116^{poly} and HCT116^{norm} (15 and 5.8 μM *vs.* 19 and 5.3 μM, in HCT116^{poly} and HCT116^{norm}, respectively) (data not shown).

Reduced sensitivity to chemotherapy associated with a G2-blockade has been reported previously[26,37]; specifically, the protracted presence of a drug-induced and maintained

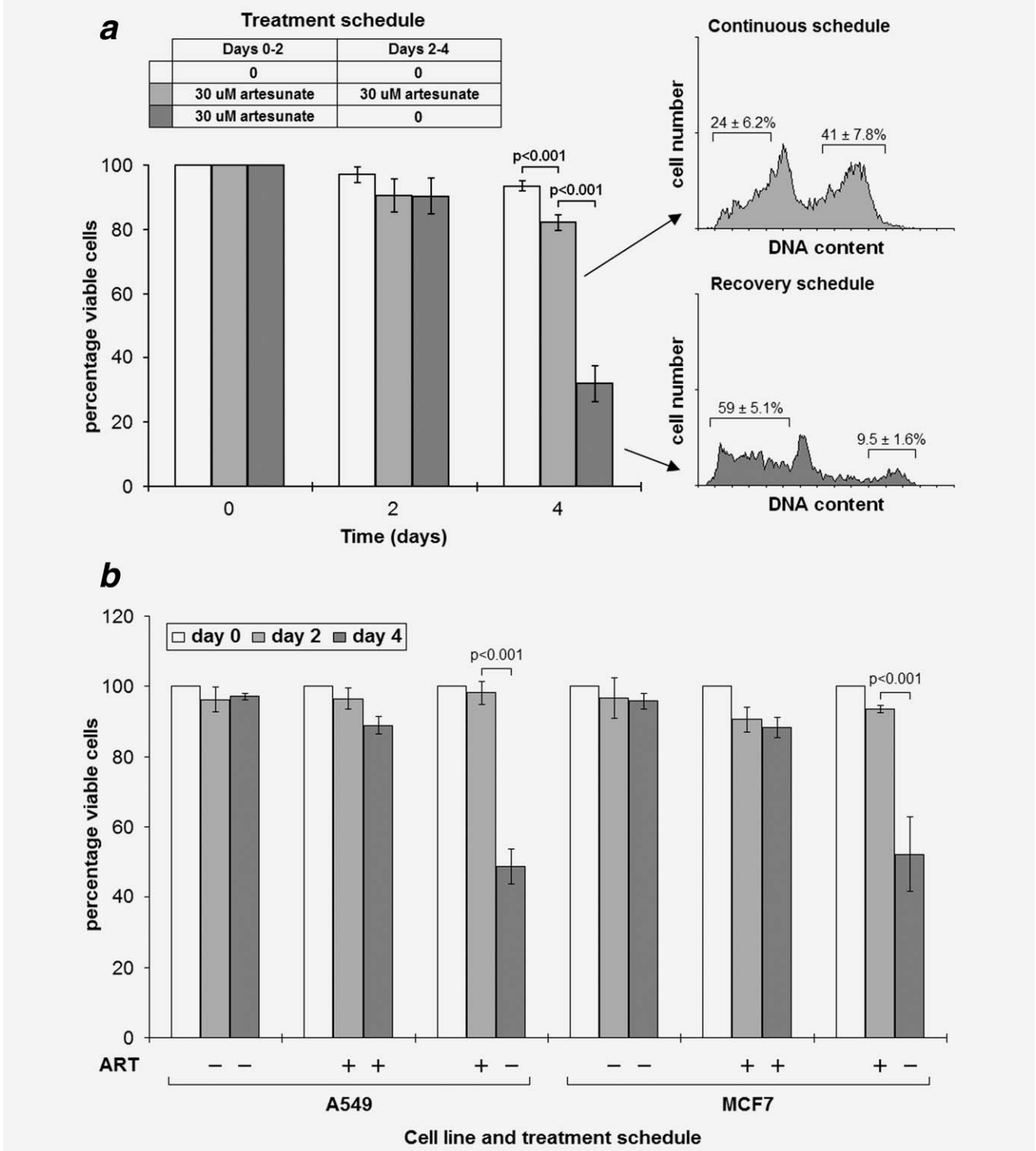


Figure 5. Effect of a drug-free period in an ART treatment schedule. (*a*) HCT116^{poly} cells were cultured with 30 μM ART for 2 days before removal of drug. Cells were then returned to fresh culture medium supplemented with or without 30 μM ART. Cell viability and cell cycle distribution were then assessed on Day 4. Results showed that culturing cells with ART for 2-days prior to returning them to drug-free medium significantly decreased cell viability, compared to culturing cells with ART for the entire 4 days. Similar treatment schedules were also tested in A549 and MCF7 cells and shown in (*b*), where each column signifies the cell viability on day 0, 2 or 4. Each of the columns represents the mean and SD of at least three separate experiments. Representative Flow cytometric histograms from three separate experiments are shown in (*a*), and include the mean percentage of cells within the sub-G1 or G2 phase.

cell cycle arrest could prevent cell death. However, cytotoxicity was restored by adapting treatment schedules to include drug-free periods, which allowed easing of the G2-block and re-engagement of cell death. For this reason, we next tested the effect of a washing step in our treatment schedules to see if sensitivity to ART could be improved in HCT116^{poly} cells. Results showed that allowing cells to grow in drug-free medium following an initial exposure to ART resulted in a significantly greater level of cytotoxicity compared to cells maintained in ART for the same duration. Furthermore, these results were subsequently recapitulated in A549 and MCF7 cells, where the removal of ART from treated cells and maintenance in drug-free medium resulted in significant reductions in cell viability.

The idea of combining drugs in therapeutic regimens is to achieve an overall effect that is greater than the sum of the individual effects of each agent.[21] This is particularly important as a number of novel therapeutic modulators appear to be ineffectual cytotoxic agents on their own, with activity greatest when utilised with a partner. For example, drugs that interfere with PI3-kinase at the level of AKT do not cause cell death directly but instead reduce resistance to other cytotoxic agents.[38,39] Furthermore, the possibility of combining drugs without a loss of effect (antagonism) would be clinically worthwhile, as a similar level of activity could be achieved at lower doses. The incidence of drug-mediated adverse events could also be minimised because of this dose reduction.

Drug combinations that involve ART have been reported *in vitro*, which show value in this approach, both as a sensitising agent to chemotherapy in solid tumours,[14,24] and as a synergistic partner with doxorubicin in leukaemia.[7] So, we next explored the value of using ART in combination with the cytotoxic agents GEM and OXP by using median-effect algorithms to generate CI-values, which allowed for the assessment of the nature of any interactions between ART and cytotoxic agent. Results showed interactions to be additive in nature as CI-values were hovering around 1, and suggested GEM and OXP in combinations did not antagonise the functions of each other. Indeed, further analysis showed that to achieve ~50% cell kill in HCT116^{norm} cells, 1.9 μM ART or 4.0 μM OXP would be required if used separately; compared to just 0.65 μM and 1.6 μM for ART and OXP, respectively, when used simultaneously.

In addition to conventional chemotherapies, we also combined ART with the immunomodulatory drug LEN.[40] Our laboratory has been actively exploring the activities of this drug and others related to it for nearly 10 years, and with other research groups, have noted similarities in the activities between ART and LEN. Both agents are multimodal and efficacious in their disease type. Both are immune modulators, antiangiogenic and antimetastatic, and affect cells at the level of intracellular signalling such as MAPK/ERK, NF-κB and p21$^{waf1/cip1}$.[19] These pathways are examples of some that are commonly dysregulated in neoplasia; hence, drugs with a diverse repertoire of activity on intracellular processes such as those mentioned, would be therapeutically worthy.[21] Our results showed that the activity of ART, as indicated by IC50-values, was enhanced by the addition of a low concentration of LEN, which had no effect on cell number and proliferation. For example, in A549 cells, the IC50 of ART in the presence of 1 μM LEN was reduced by 48% (IC50: 5.1 ± 0.61 *vs.* 9.8 ± 1.7 in cells cultured with ART alone; $p < 0.001$). Parenthetically, this model, which involved modifying the activity of the primary cytotoxic agent with a subactive concentration of the modulating drug-partner, has been proposed and described previously in combination cases where one drug is ineffectual with regards to affecting the final readout.[12,21,25]

This study has reinforced a possible application for ART in a cancer therapy setting, not only as a single agent but also in combination with some chemotherapy agents. This *in vitro* study highlights impressive antiproliferative activity in a small panel of cell lines, and complements an ongoing randomised tolerability and efficacy study of oral ART in patients with colorectal carcinoma. The *in vitro* study also describes the effects of ART on the cell cycle, and presents data that shows restoration of cytotoxicity in an ART-resistant cell by adopting a pulsed-schedule. The mechanism underlying the combinatorial interaction, and indeed the mechanism of ART action in cancer *per se* is still not fully elucidated; however, it is becoming apparent that these involve or are associated with modulation of cell cycling proteins. This work is ongoing and currently forms the basis of further studies.

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