Original Article

A Randomised, Double Blind, Placebo-Controlled Pilot Study of Oral Artesunate Therapy for Colorectal Cancer

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A B S T R A C T

Background: Artesunate is an antimalarial agent with broad anti-cancer activity in in vitro and animal experiments and case reports. Artesunate has not been studied in rigorous clinical trials for anticancer effects.

Aim: To determine the anticancer effect and tolerability of oral artesunate in colorectal cancer (CRC).

Methods: This was a single centre, randomised, double-blind, placebo-controlled trial. Patients planned for curative resection of biopsy confirmed single primary site CRC were randomised (n = 23) by computer-generated code supplied in opaque envelopes to receive preoperatively either 14 daily doses of oral artesunate (200 mg; n = 12) or placebo (n = 11). The primary outcome measure was the proportion of tumour cells undergoing apoptosis (significant if >7% showed Tunel staining). Secondary immunohistochemical outcomes assessed these tumour markers: VEGF, EGFR, c-MYC, CD31, Ki67 and p53, and clinical responses.

Findings: 20 patients (artesunate = 9, placebo = 11) completed the trial per protocol. Randomization groups were comparable clinically and for tumour characteristics. Apoptosis in >7% of cells was seen in 67% and 55% of patients in artesunate and placebo groups, respectively. Using Bayesian analysis, the probabilities of an artesunate treatment effect reducing Ki67 and increasing CD31 expression were 0.89 and 0.79, respectively. During a median follow up of 42 months 1 patient in the artesunate and 6 patients in the placebo group developed recurrent CRC.

Interpretation: Artesunate has anti-proliferative properties in CRC and is generally well tolerated.

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1. Introduction

Colorectal cancer (CRC) contributes 9–10% of the annual global cancer burden in men (746,000 cases) and women (614,000 cases) (Ferlay et al., 2012). In the UK, 110 new cases are diagnosed daily, with older patients particularly at risk of death (UK CR, 2014) and with >50% of newly diagnosed cases having locally advanced disease (T3/T4). Resection is the only curative treatment for non-metastatic CRC but this has to be combined with neo-adjuvant chemo- and/or radio-therapy, to downstage more advanced presentations.

Prognosis with best available treatments does not increase disease free or overall survival beyond ~60% at 5 years after diagnosis. For most patients, access to advanced treatment modalities is lacking, too expensive to be widely available, or associated with significant morbidity thereby further compromising their survival. There is therefore a continuing and urgent need to develop new, cheap, orally effective and safe CRC therapies. One approach is to study existing drugs that already have some anticancer properties in experimental settings, and to assess their safety and efficacy in in vivo studies.

Artesunate is derived from artemisinin, which is extracted from Artemisia annua L. and is a widely used antimalarial that can be administered by oral, rectal and parenteral routes (Comes et al., 2009; Kremsner and Krishna, 2004; Kremsner et al., 2012; Nealon et al., 2002; Hien et al., 1994, 1992; Jiang et al., 1982). Soon after the isolation of artemisinin by a Chinese government’s programme, the anticancer properties of artemisinins were first reported (Efferth et al., 2007;
 manufacture tablets were manufactured by MPF in The Netherlands under a manufacturing licence in accordance with EU cGMP certified by Dafra Pharma (Belgium). Study medication was packaged, labelled and certified by B&C CliniPack (Belgium) and was in packs sizes of 30 × 100 mg and was received, stored and dispensed by the Pharmacy at St George’s Healthcare NHS Trust.

The dose of artemunate for the study was 200 mg orally, daily for fourteen days, with medication stopped 48–72 h prior to surgery.

Medication was provided in blister packs with one patient box provided 14 doses, sufficient for the duration of the study.

There was no delay in surgery if patients entered into this study, nor any other change in clinical management, and the 62 day rule (requiring treatment within this time period after confirmation of diagnosis) was strictly adhered to.

2.7. Outcomes

The primary endpoint of the trial was the presence or absence of significant apoptosis in the epithelial cells of the tumour specimen defined as >7% of cells with apoptotic features.

Secondary outcomes included seven immunohistochemical stains applied to the paraffin-embedded tumour specimens and quantified in both epithelial cells and fibroblasts: vascular endothelial growth factor (VEGF), c-MYC status and EGF-receptor status; microvessel density determining the quantity of the cluster of differentiation 31 (CD31) protein; proliferative activity assessed with Ki67 staining and p53 tumour suppressor protein expression. Each stain in each patient was generally evaluated in 6 microscopic areas with a semiautomatic system (in a few cases 7 or 8 areas were evaluated, and in some – especially for fibroblasts – measurements less than 6 or no areas could be evaluated).

2.8. Blood Samples

Three blood samples were taken: (1) at baseline, (2) after one week of medication (following protocol amendment for enhanced safety monitoring) and (3) after ending the two week medication (just before surgery). In each sample the safety measures included assay of potassium, sodium, creatinine, urea, albumin, alkaline phosphatase, ALT, bilirubin, haemoglobin, platelet count and white cell count. Carcinoembryonic antigen (CEA), was monitored where available in patients at baseline and after randomization.

2.9. Secondary Outcomes

These were measures of safety and tolerability (both clinical and laboratory) according to conventional criteria assessed by comparing baseline blood test results and those during or after treatment and anti-cancer efficacy (with markers described above).

2.9.1. Changes to Outcomes

There were no changes to predefined endpoints.

2.10. Sample Size

An indicative sample size calculation, given the pioneering nature of this pilot study, was carried out on the primary outcome before starting the trial based on the assumption that colorectal cancer is unlikely to exhibit significant apoptosis if untreated. Most patients in the placebo group (more than 95%) were anticipated to have less than 7% of cells with apoptotic features. The majority of patients (greater than 60%) in the artemunate group were anticipated to have significant apoptosis. This large difference was derived from published baseline estimates of apoptotic indices (Yamamoto et al., 1998; Ikenaga et al., 1996; Bendardaf et al., 2003). With equal group sizes a sample size of 211 was estimated to have 80% power and accepting a Type I error of 5% for superiority, bearing in mind that in most pilot studies the aim is to demonstrate proof-of-concept (Arain et al., 2010) rather than exclusively test a hypothesis.
2.11. Randomisation

Subjects were randomised to receive either artesunate or placebo in equal numbers. Randomisation was performed using a computer-generated code, and results supplied in opaque and sealed envelopes by Dafra Pharma. After enrollment and allocation of the next study number in the series, participants were given their randomization pack by a pharmacist. Copies of the key to the randomization codes were held by the Clinical Trials Pharmacist only and allocation and concealment steps were performed in Belgium. The code was not opened to investigators, patients, data collectors until the data collection ended, histological results had been analysed and datasets were locked.

2.12. Sequence Generation and Allocation Concealment Mechanism

Study medications were pre-packed in blister packs and consecutively numbered for each participant according to the randomisation schedule. Each participant was assigned an order number once they had consented to the study and after eligibility checks, and they received the medication pack with the corresponding randomization number.

2.13. Blinding

It became necessary to unblind the allocation to 2 participants during the course of the study at the request of the MHRA after receipt of notification of Adverse Events. Blinding was maintained for all investigators and codes were supplied by the Sponsor’s office (SGUL) to the MHRA.

2.14. Data Collection and Structure

Immunohistopathological data generated multiple measurements per individual as the number of slides differed according to the sizes of the tumours. Each measurement represents an estimate of staining of cancer cells found on a slide with 0 denoting no staining observed on a section. Therefore, the dataset inherits a hierarchical structure with patients at level one and within individual measurements as level two. With the exception of the three individuals (Fig. 1) there are no records considered missing for statistical analysis.

2.15. Exploratory Statistical Analysis

The nature of all data variables have been graphically assessed and summarised accordingly with means/medians standard deviation for continuous data or proportions for binary data. Correlations were explored with Spearman’s coefficient.

2.16. Immunochemistry Results and Inferential Data Analysis

A random effects (variance components) model was employed for immunochemistry data to capture their variabilities correctly, given their inherent hierarchical structures.

Patients were followed-up with CEA measurements every 6 months and annual CT scans for disease recurrence. Time since surgery to the first disease recurrence has been modelled with survival analysis. Patient CRC06 has been included in survival analysis and although there were no samples obtained for immunochemistry, the patient was known to have survived. Patient CRC13 was initially randomised

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**Fig. 1.** Patient flow diagram. Assessment for eligibility was recorded after 6 patients had been randomised, so that these added to the 17 patients randomised after screening give the total number of randomised patients (n = 23).
to artesunate but was included in the survival analysis as placebo as s/he did not receive any drug (as per protocol analysis). Patient CRC21 was deemed as missing and sensitivity analysis including them in either artesunate or placebo groups is provided. The Cox proportional hazard's (PH) model has been applied to investigate the hazard ratio of disease recurrence for artesunate compared with placebo, and their pointwise 95% confidence intervals are provided for each treatment group.

2.17. Statistical Inference

Model based Bayesian analysis and classical frequentist approaches have been applied as appropriate. Frequentist statistical significance is conventionally associated with p-values less than 0.05 with uncertainty of parameters assessed by the 95% confidence intervals (CI). Parameter estimates in Bayesian inference are summarised by their posterior means and the corresponding 95% credible intervals (CrI). Initially, no prior knowledge was assumed for the parameter that quantifies the treatment effect, i.e., the difference between the group means in terms of immunohistochemistry measurements and the inference that has been drawn. If prior information exists, then it is appropriate to assess how the parameter values change based on this evidence. Ki67 and CD31 were the only stains with prior anti-CRC information available (Li et al., 2007; Jansen et al., 2011). A sensitivity analysis was conducted with Ki67. Statistical software used included OpenBUGS (Thomas et al., 2006) STATA (StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX: StataCorp LP) and R (Team RDC, 2011).

3. Results

3.1. Participant Flow

Fig. 1 summarises patient flows. 12 patients were randomised to receive artesunate and 11 to placebo. 2 patients did not receive artesunate despite randomization (one travelled for surgery outside the UK and could not be followed up, and the drug expiry date for the relevant batch had been reached when the other patient attended). One artesunate recipient could not be evaluated for the primary endpoint

Table 1
Baseline demographic, clinical and laboratory characteristics.

|                   | Artesunate          | Placebo            | Numbers* (artesunate/placebo) |
|-------------------|---------------------|--------------------|------------------------------|
|                   | N = 12              | n = 11             |                              |
| Demographics      | Mean (SD)           | Mean (SD)          |                              |
| Age (y)           | 69 (11)             | 66 (14)            | 12/10                        |
| Gender (% Female) | 0.58                | 0.64               |                              |
| Ethnicity (% Caucasians) | 0.83              | 0.82               |                              |
| Height (m)        | 1.70 (0.12)         | 1.62 (0.08)        | 10/9                         |
| Weight (kg)       | 74 (16.7)           | 75 (66.85)         | 11/10                        |

Biochemistry

|                   | Artesunate          | Placebo            |                              |
|-------------------|---------------------|--------------------|------------------------------|
| Sodium (mmol/L)   | 140 (1)             | 139 (2.28)         | 11/11                        |
| Albumin (g/L)     | 38 (5.7)            | 36 (5.7)           | 11/11                        |
| ALT (UL)          | 23 (8)              | 24 (10)            | 11/11                        |
| Bilirubin (μmol/L)| 9 (3)               | 8 (3)              | 9/11                         |
| Creatinine (μmol/L)| 73 (23)           | 65 (15)           | 9/11                         |
| Urea (mmol/L)     | 5 (2)               | 5 (1)              | 9/11                         |

Haematology

|                   | Artesunate          | Placebo            |                              |
|-------------------|---------------------|--------------------|------------------------------|
| Haemoglobin (g/dL)| 11.5 (2)            | 12.2 (2)*          | 9/11                         |
| White Cell count (/L)| 5.3 (2.8)      | 6.7 (2)            | 9/11                         |
| Platelet Count (/L)| 370000 (20000)*   | 31300 (78000)      | 8/11                         |

Dukes’ stage

|       | Artesunate | Placebo |                              |
|-------|------------|---------|------------------------------|
| A     | 2          | 2       |                              |
| B     | 5          | 3       |                              |
| C1    | 2          | 6       |                              |
| C2    | 1          | 1       |                              |

* Numbers of patients contributing to each value are indicated in the last column.

Table 2
Predicted “grand means” of immunohistochemistry results. Results are presented on a linear scale by treatment groups with their 95% credible intervals following a Bayesian analysis which takes into account the variability within individual measurements as well as that of between different individuals in the cluster. The probability of an effect is the probability that the difference between the grand means in the two groups (artesunate-placebo) is greater than 0 (and is not a p-value). For Ki67 all corresponding informative priors are presented in table 1. Parameter values change based on this evidence. Ki67 and CD31 were the only stains with prior anti-CRC information available (Li et al., 2007; Jansen et al., 2011). A sensitivity analysis was conducted with Ki67. Statistical software used included OpenBUGS (Thomas et al., 2006) STATA (StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX: StataCorp LP) and R (Team RDC, 2011).

| Marker   | Artesunate Mean (95% CrI) | Placebo Mean (95% CrI) | P of an effect | Difference or change | Sensitivity analysis (prior) |
|----------|---------------------------|------------------------|----------------|----------------------|------------------------------|
| Epithelial |                           |                        |                |                      |                              |
| c-MYC    | 45 (27, 53)               | 43 (27, 64)            | 0.59           | 3.6 (−22, 26)        | N (0, 1000) Non-informative   |
| CD31     | 8 (3.12)                  | 5 (2.9)                | 0.79           | 2.6 (−4, 9)          | N (0, 100) Vaguely informative|
| EGFR     | 32 (12.58)                | 27 (9.45)              | 0.66           | 5.4 (−24, 32)        | N (0, 10) Informative         |
| p53      | 25 (5.45)                 | 18 (3.35)              | 0.72           | 7 (−20, 30)          | N (−15, 100) Non-informative  |
| Tunel    | 18 (4.32)                 | 20 (7.32)              | 0.4            | −2 (−20, 17)         | N (−15, 10) Vaguely informative|
| VEGF     | 38 (21, 54)               | 43 (40, 75)            | 0.45           | −5 (−28, 17)         | N (−15, 10) Informative       |
| K67*     | 33 (13, 52)               | 49 (31, 66)            | 0.11           | −16 (−42, 10)        | N (0, 1000) Non-informative   |
| Fibroblast |                           |                        |                |                      |                              |
| c-MYC    | 7 (−7, 21)                | 19 (7,32)              | 0.10           | −12 (−30, 7)         | N (0, 100) Vaguely informative|
| CD31     | 13 (−2, 26)               | 11 (0.23)              | 0.58           | −1 (−17, 19)         | N (−15, 10) Non-informative  |
| EGFR     | 0.1 (−0.5, 0.7)           | 0.4 (0.0, 0.9)         | 0.21           | −0.3 (−1, 1)         | N (−15, 10) Non-informative  |
| p53      | 0.7 (−0.8, 2.1)           | 0.9 (−0.3, 2)          | 0.40           | −0.2 (−2.1, 12)      | N (−15, 10) Vaguely informative|
| Tunel    | 3 (0.5)                  | 4 (1.6)                | 0.31           | 0.8 (−4.3)           | N (−15, 10) Informative       |
| VEGF     | 0.2 (0.02, 0.5)           | 0.2 (0.1, 0.3)         | 0.52           | 0.02 (−0.3, 0.3)     | N (−15, 10) Informative       |
because no tumour was identified by histology after operation. Recruitment ended after the planned numbers were randomised.

3.2. Baseline Data

Baseline demographic and clinical characteristics are summarised in Table 1. There is comparability between groups, including for Dukes’ staging.

3.3. Primary Outcome

55% of placebo recipients and 67% of artesunate treated patients achieved the primary outcome (patients in whom the proportion of apoptotic cells was >7%). In designing this trial, it was assumed that only ≤1/11 patients receiving placebo would have >7% of tumour cells displaying apoptosis. The unpredicted higher baseline values in placebo recipients precluded detection of an artesunate effect in the primary outcome. Staining results for Tunel as a continuous variable are in Table 2.

3.4. Secondary Outcomes

3.4.1. Immunohistochemistry Analyses

Table 2 presents analysis of immunohistochemistry results. A random effects model scaled linearly provides the posterior distributions of the group means, their 95% CrI and their estimated differences between groups. The estimated posterior distributions of differences between treatment groups lie on both sides of 0 for most measurements suggesting that the two treatment groups do not differ markedly for most analysed markers.

Interestingly, the probability of a reduction in Ki67 staining after artesunate is 89–92% (Table 2; a result that is 1–0.11, because Ki67 is downregulated) using a non-informative prior on the Ki67 difference between groups and resulting in a posterior difference of −16 (−42, 10) for treatment. A similar result is confirmed if the confidence around the parameter value is increased as illustrated in Fig. 2a. With an optimistic informative prior for artesunate of −15 (N (−15,10)), the probability of an artesunate effect increases to 97% (1–0.03; Table 2), by altering the parameter distributions as summarised in Fig. 2b.

To complete this analysis, we included a skeptical informative prior for this parameter (N (0, 10)). Despite this, the probability of an artesunate effect remains at a probability of 0.77 with a true mean value estimated at −16 (−22.10; Table 2). CD31 also provided a high probability (0.79) for a treatment effect. Representative immunostainings are shown in Fig. 3.

3.4.2. Survival Analysis

During a median follow up of 42 months, there were 6 recurrences in the placebo group and 1 recurrence in an artesunate recipient. A probability (0.79) for a treatment effect. A similar result is confirmed if the confidence around the parameter value is increased as illustrated in Fig. 2a. With an optimistic informative prior for artesunate of −15 (N (−15,10)), the probability of an artesunate effect increases to 97% (1–0.03; Table 2), by altering the parameter distributions as summarised in Fig. 2b.

To complete this analysis, we included a skeptical informative prior for this parameter (N (0, 10)). Despite this, the probability of an artesunate effect remains at a probability of 0.77 with a true mean value estimated at −16 (−22.10; Table 2). CD31 also provided a high probability (0.79) for a treatment effect. Representative immunostainings are shown in Fig. 3.

3.4.3. CEA Levels

Six artesunate and 4 placebo recipients had CEA levels measured before and after trial medication (and before resection, classified as reduced, stable or increased). No patients with artesunate had increased CEA levels whereas 3 patients in the placebo group had increased values (p = 0.03, Fisher’s exact test).

3.5. Adverse Events

Six patients (26% for the ITT population) had adverse events (2 severe, Table 3 and Supplementary Table 1). Two adverse events possibly related to study drug are described in detail. In the remaining 4 cases, 2 complications (anastomotic leaks after surgery) were considered unlikely to be related to artesunate, and one case of iron-deficiency anaemia (with no neutropaenia) was attributed to underlying disease. There was one report of nausea. Detailed descriptions of 2 cases of neutropaenia are given below and illustrated in Fig. 5.

CRC04: An 81 yo 51 kg female presented with anaemia and a change in bowel habit, and was discovered to have a large, annular ascending colon, polypoidal, carcinoma that was not producing obstruction on colonoscopy. She was randomised to receive artesunate. There was no evidence of metastatic spread on staging scans (CEA = 3 μg/L). Her mid-treatment review was unremarkable. She returned for surgery and was found to be anaemic and neutropenic (Fig. 4a). She was transfused, making this a Grade 3 adverse event according to CTCAE criteria (v4.0; http://
CRC07: A 79 yo 50 kg lady presented with anaemia, rectal bleeding and a change in bowel habit in the preceding few months. She had a history of endometrial carcinoma and underwent a total abdominal hysterectomy and bilateral salpingo-oophorectomy followed by radiotherapy 11 years before. Colonoscopy confirmed an adenocarcinoma with impassable stricture at the splenic flexure. Staging CT scan of the chest, abdomen and pelvis excluded metastasis. She was randomised to receive artesunate. Her CEA was 212 μg/L but fell steeply following artesunate (to a nadir of 56 μg/L, Fig. 4b) and no other intervention. She had a persistent thrombocytosis, but she developed anaemia and leucopenia, which...
After 3 years of follow up she is confirmed to be symptom and disease-free and continues to lead an independent life.

The recurrence-free survival probability was also higher after artesunate compared with placebo (at 3 years 0.89 compared with 0.5; Fig. 3) although confidence intervals for these estimates overlap (HR 0.16, p = 0.091, Supplementary Table 1) because of the small numbers of patients and therefore events included in this study. Till this analysis, there have been no deaths in artesunate recipients (despite some patients having relatively poor prognosis), and 3 deaths in placebo recipients.

Two patients who were at the lower weight limit for inclusion in this study (50 kg, giving an effective dose of 4 mg/kg of artesunate/day) developed leucopenia (Fig. 4). In one case this reversed shortly after stopping artesunate, whereas in the other G-CSF may have hastened recovery. Bone-marrow examination suggested a toxic effect of artesunate. These findings are consistent with the recent observations in malaria of a dose-dependent neutropenia with artesunate (>4 mg/kg) (Bethell et al., 2010), although bone marrow examinations have not been carried out before. We instituted mid-treatment monitoring for neutropenia after observations on malaria but did not note this complication in other patients. Artesunate associated leucopenia may be dose-dependent in cancer patients as it is in malaria, and although delayed haemolysis has been observed after artemisinin use (Rolling et al., 2014, 2012) it was not a complication in our patients. In future studies, it may be safer to restrict daily dose of artesunate to ∼4 mg/kg and to monitor for haematological complications. A recent publication on an artesunate dose-finding study in metastatic breast cancer disease suggests that 200 mg once a day can be tolerated for up to 3 weeks (Ericsson et al., 2014).

Lever recurrence was commonest in our patients, followed by peritoneal and ovarian sites, suggesting that seeding is mainly haematogenous and trans-peritoneal. As patients had clear circumferential and longitudinal margins at surgery, and detectable metastases were not identified at

### Table 3

| Study number | Event                  | Due to pre-existing illness | Related to study drug | Serious? | Study treatment          | Outcome  |
|--------------|------------------------|----------------------------|-----------------------|----------|--------------------------|----------|
| CRC 004      | Neutropaenia and anaemia | No                         | Possibly              | N        | Stopped                  | Resolved |
| CRC 007      | Neutropaenia and anaemia | No                         | Possibly              | Y        | Stopped                  | Resolved |
| CRC 017      | Nausea, but no vomiting | No                         | Possibly              | N        | Continued unchanged      | Resolved |
| CRC 018      | Anastomotic leak        | No                         | Not related           | Y        | Stopped                  | Resolved |
| CRC 019      | Anastomotic leak        | No                         | Unlikely              | Y        | Stopped                  | Resolved |
| CRC 022      | Anaemia                 | Yes                        | Unlikely              | N        | Continued unchanged      | Resolved |
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Author contributions
SK and HJ conceived the study and designed it together with PGK, DK and TE; ICS carried out statistical analysis, SG, MC, DK and SK recruited and managed patients; HK managed the study; CF provided clinical histopathological analysis; TE and MEMS analysed immunohistochemistry; SK wrote the first draft to which all authors contributed to produce the final version. No author declares a conflict of interest.

Appendix A. Supplementary Data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2014.11.010.

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