Heterogeneity of chemosensitivity of colorectal adenocarcinoma determined by a modified *ex vivo* ATP-tumor chemosensitivity assay (ATP-TCA)

Pauline A. Whitehouse[a], Louise A. Knight[a], Federica Di Nicolantonio[a], Stuart J. Mercer[a], Sanjay Sharma[a] and Ian A. Cree[a] on behalf of the Portsmouth Colorectal Cancer Multidisciplinary Team

Advanced colorectal cancer (CRC) has a poor prognosis with a 5-year survival of only 5% despite treatment with chemotherapeutic agents. Response rate and overall survival varies little between the commonly used single agents, although combinations achieve better outcomes. It is well established that considerable heterogeneity exists between cancers of the same tissue type, but it has been difficult to establish this for CRC. We therefore investigated the heterogeneity of chemosensitivity in CRC using a modified version of the *ex vivo* ATP-tumor chemosensitivity assay (ATP-TCA) capable of handling infected tumor tissue. Fifty-three specimens of primary solid or malignant effusions of CRC were tested, of which 46 (87%) were evaluable. There were considerable differences in sensitivities between individuals. The most active single cytotoxic agents in the assay were identified as 5-fluorouracil, irinotecan and mitomycin C (MMC). Cells were exposed to combinations of drugs added simultaneously at the same concentrations tested as single agents. All drug combinations achieved greater growth inhibition than drugs used alone. MMC + gemcitabine was found to be the most effective combination in 83% of specimens. The ATP-TCA has previously been shown to be a good predictor of response to chemotherapy in other tissue types. The degree of heterogeneity demonstrated from these results suggests that the ATP-TCA could be used to identify patients who might benefit from specific chemotherapeutic agents alone or in combination. *Anti-Cancer Drugs* 14:369–375 © 2003 Lippincott Williams & Wilkins.

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[a]Translational Oncology Research Centre, Queen Alexandra Hospital, Portsmouth PO6 3LY, UK.

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Correspondence to I. Cree, Translational Oncology Research Centre, Department of Histopathology, Queen Alexandra Hospital, Cosham, Portsmouth PO6 3LY, UK.
Tel: +44 23 92 286000; fax: +44 23 92 286379;
e-mail: ian.cree@port.ac.uk

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Introduction

Colorectal adenocarcinoma (CRC) is the second most common cancer in both men and women, and is the second leading cause of cancer-related death in the UK [1]. Although 70% undergo potentially curative surgery, half of all patients present with or develop advanced local disease or metastases. Advanced CRC has a poor prognosis with a median survival of only a few months (6–15 months) despite chemotherapy.

5-Fluorouracil (5-FU), as single agent or in combination, has been the mainstay of medical treatment for CRC for over 40 years. This is due to its relatively low toxicity and the inability of newer drugs, used as single agents, to achieve significantly better response rates. Attempts have been made to ameliorate the effect of 5-FU by biochemical modulation and route of administration. Modulation with leucovorin is standard treatment even though a meta-analysis found it increased response rate, but not overall survival [2]. Continuous infusion significantly increases response rate, but produces only a modest increase in overall survival compared to bolus administration [3]. As a result, a variety of 5-FU regimens are available to the clinician. In the UK, the current National Institute for Clinical Excellence guidance on the treatment of advanced CRC advocates that 5-FU with leucovorin should remain as first-line treatment [4]. The response rates and overall survival achievable with this combination are 18–22% and 12–15 months, respectively [5,6]. In combination with 5-FU, oxaliplatin and irinotecan have produced response rates of up to 50%, but this has not translated into better overall survival and, at present, 5-FU with leucovorin remains the standard against which others are judged [7,8].

Currently, much effort is being spent studying the molecular biology of CRC with an aim to determine markers useful in the prediction of outcome. New

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molecularly targeted drugs are appearing, but these have yet to make a clinical impact. Such drugs require that the target is present and functionally abnormal in the tumor, and that interference with the target alters the biology of the cancer. Tumors show heterogeneity of genotype and phenotype, and such heterogeneity in colorectal tumors almost certainly affects response to 5-FU and other cytotoxic agents [9]. Predictive assays based on thymidylate synthase levels show some promise, but cellular assays have largely been ignored due to low evaluability rates and technical problems, particularly infection, which is common in tumor-derived tissue [10,11]. However, recent technical developments have produced assays, such as the ATP-tumor chemosensitivity assay (ATP-TCA), which has high evaluability rates with solid tumors and produces interpretable results in more than 90% of tumors tested [12,13]. The results correlate well with outcome in patients with a sensitivity of 95% for predicting those who will respond to primary treatment of stage III ovarian cancer [14]. The use of this assay has been shown to double progression-free survival and overall survival in a case-control intervention study in recurrent ovarian carcinoma [15].

We performed this study to determine the degree of heterogeneity of chemosensitivity in colorectal adenocarcinoma as a prelude to studies of the molecular basis of resistance in tumor-derived cells and the potential use of this assay to guide therapy. We also wished to counter any ATP-TCA technical problems, particularly the use of infected tumor material.

Materials and methods

Tumor specimens

A total of 53 specimens were studied and 46 of these produced evaluable results (87%). Forty-four were from patients undergoing resection of their primary colorectal adenocarcinoma (of all pathological stages) and two were ascites/pleural aspirates in patients with metastatic disease. All seven tumors that failed showed infection during short-term cell culture. Due to initial problems of plate infection of the colorectal samples, we added further antibiotics to our culture medium. In order to ensure the antibiotics did not alter the sensitivity of the cytotoxic drugs, we performed experiments in parallel using medium with and without antibiotics (amphotericin B and metronidazole).

Of the solid tumors, 34 were colonic, nine were rectal and two were peritoneal biopsies. The median age of the patients was 70 years (range 39–86). Of the rectal cancers, two patients had received neoadjuvant chemoradiotherapy. Local ethics committee approval was obtained and informed consent gained from all patients. Biopsies were taken from the luminal surface of resection specimens by a pathologist or surgeon, ensuring histopathological diagnosis and staging were not compromised.

ATP-TCA

The ATP-TCA was performed as previously described [12]. Solid tumor material was minced and dissociated in 1.5 mg/ml collagenase (Sigma, Poole, UK; C8051) overnight. The samples were purified using Ficoll-Hypaque (Sigma; 1077-1) to remove red blood cells and excess debris, and resuspended in serum-free complete assay medium (CAM; DCS Innovative Diagnostik Systeme, Hamburg, Germany) containing penicillin–streptomycin (Sigma), gentamicin (Sigma), and additional amphotericin B (Sigma) and metronidazole (Rhône Poulenc Rorer, Eastbourne, UK). The cells were then counted and viability assessed by the Trypan blue exclusion method. The final cell suspension was made up to a concentration of 200 000 cells/ml for solid tumors and 100 000 cells/ml for malignant effusions. Round-bottomed polypropylene 96-well plates (Corning-Costar, High Wycombe, UK) were prepared with CAM and cytotoxic drugs at six dilutions (6.25–200%) of the test drug concentration (TDC) in triplicate. The TDC for each drug has previously been calculated from pharmacokinetic and response data (Table 1) [12]. The drugs were stored appropriately and made up according to the manufacturer's instructions [16]. Dilutions were prepared in the plates from freshly made up 800% TDC drug solutions. Combinations of drugs were made by adding both drugs each at their 800% TDC. Each plate contained two rows of internal controls: a maximum inhibitor (MI) that kills all the cells giving a zero ATP count and a medium only (MO) of CAM without any drugs. After 6 days incubation at 37°C at 100% humidity in 5% CO_2, the cells were lysed with a detergent-based Tumor Cell Extraction Reagent (DCS Innovative Diagnostik Systeme) and the ATP content of each well measured using the luciferin–luciferase system in a microplate luminometer (MPLX; Berthold, Pforzheim, Germany).

Drugs

Irinotecan (CPT-11) is converted to its inactive metabolites by cytochrome P450, and to its active metabolite, SN38, by carboxyesterases in the liver, blood and tumors.

Table 1 Drugs tested and their 100% TDC as used in the *ex vivo* ATP-TCA

Drug/combination	100% TDC (μg/ml)
5-FU	45
Irinotecan	100
Oxaliplatin	5
MMC	0.7
Gemcitabine	12
SN38	0.06
5-FU + oxaliplatin	45 + 5
5-FU + irinotecan	45 + 100
5-FU + MMC	45 + 0.7
MMC + gemcitabine	0.7 + 12
Oxaliplatin + gemcitabine	5 + 12

As these enzymes are not necessarily present in our culture environment, we tested both irinotecan and SN38 in 11 samples.

Data analysis

Data was transferred directly from the luminometer to a spreadsheet (Excel 2000; Microsoft). The results are expressed as a number of indices of efficacy, including the IC_{50} and IC_{90}. The natural logarithmic sum index ($Index_{SUM}$) calculated as:

$$\text{Index} = \text{Sum}[\text{Inhibition}_{6.25+12.5+25+50+100+200}]$$

has been shown to allow simple comparison of results between drugs and tumors [17]. In addition, the area under the concentration–inhibition curve ($Index_{AUC}$) and the percentage of tumors achieving 90% inhibition was calculated.

Results

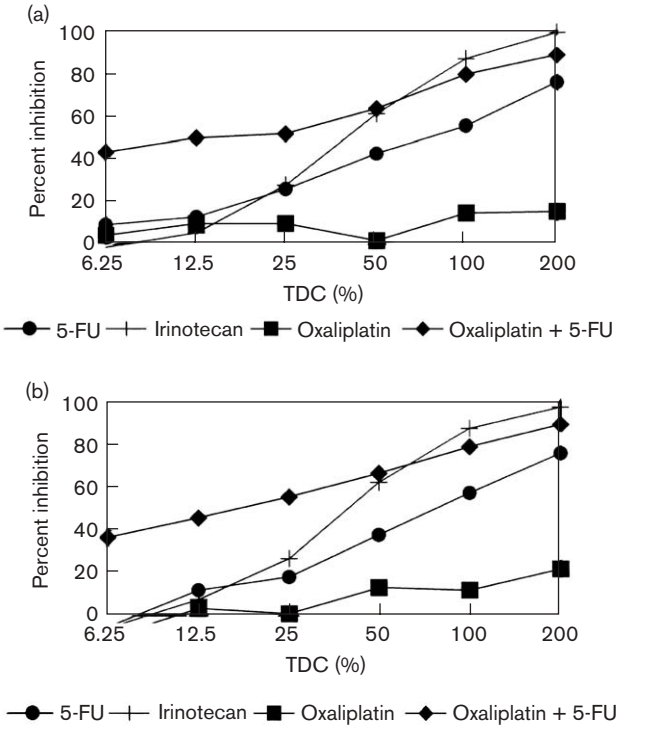
The evaluability rate (i.e. the number of tumors with interpretable results) of colorectal specimens was 87% (46 of 53). The failures were due to infection of the 96-well plates and were experienced at the beginning of the study. As a result, additional antibiotics were added to the medium to cover anaerobic and fungal infections. The addition of 2.5 μg/ml amphotericin B and 1 μg/ml metronidazole did not alter the sensitivity of the cytotoxic drugs used (Fig. 1).

For comparison between drugs and tumors, an $Index_{SUM} < 300$, representing an average 50% inhibition across all concentrations tested, was used to indicate sensitivity, as previously published [18,19]. The results show considerable heterogeneity of chemosensitivity to single agents and drug combinations between the tumors tested (Fig. 2, and Tables 2 and 3). Figure 2 shows the distribution of sensitivity of all tumors for all single agents and combinations tested.

All single agents tested, except oxaliplatin, were active in about 50% of samples (50–59%) on the basis of the $Index_{SUM} < 300$ threshold (Fig. 3). The most active single agent tested was 5-FU, to which 59% of samples were sensitive. All drug combinations achieved greater growth inhibition than drugs used alone (73–100%). All samples were sensitive to mitomycin C (MMC) + gemcitabine and this was the most effective combination in 83% of the tumors tested (29 of 35). Eleven samples were tested with both SN38 and irinotecan. Of these 11, none were sensitive to SN38 at the concentration tested (0%), but seven were sensitive to irinotecan (64%) (Fig. 4).

Some tumors responded well to one drug or combination, while others showed no response to this and instead responded to an alternative regimen. For a limited panel of drugs and combinations (5-FU, irinotecan, oxaliplatin, 5-FU + irinotecan, 5-FU + oxaliplatin and 5-FU + MMC), five cases were sensitive to only one drug/combination and resistant to all the others tested. Of these five, two were sensitive only to 5-FU, one to 5-FU + irinotecan, one to 5-FU + oxaliplatin and one to 5-FU + MMC. Two cases were resistant to all drugs/combinations tested (4%).

Fig. 1

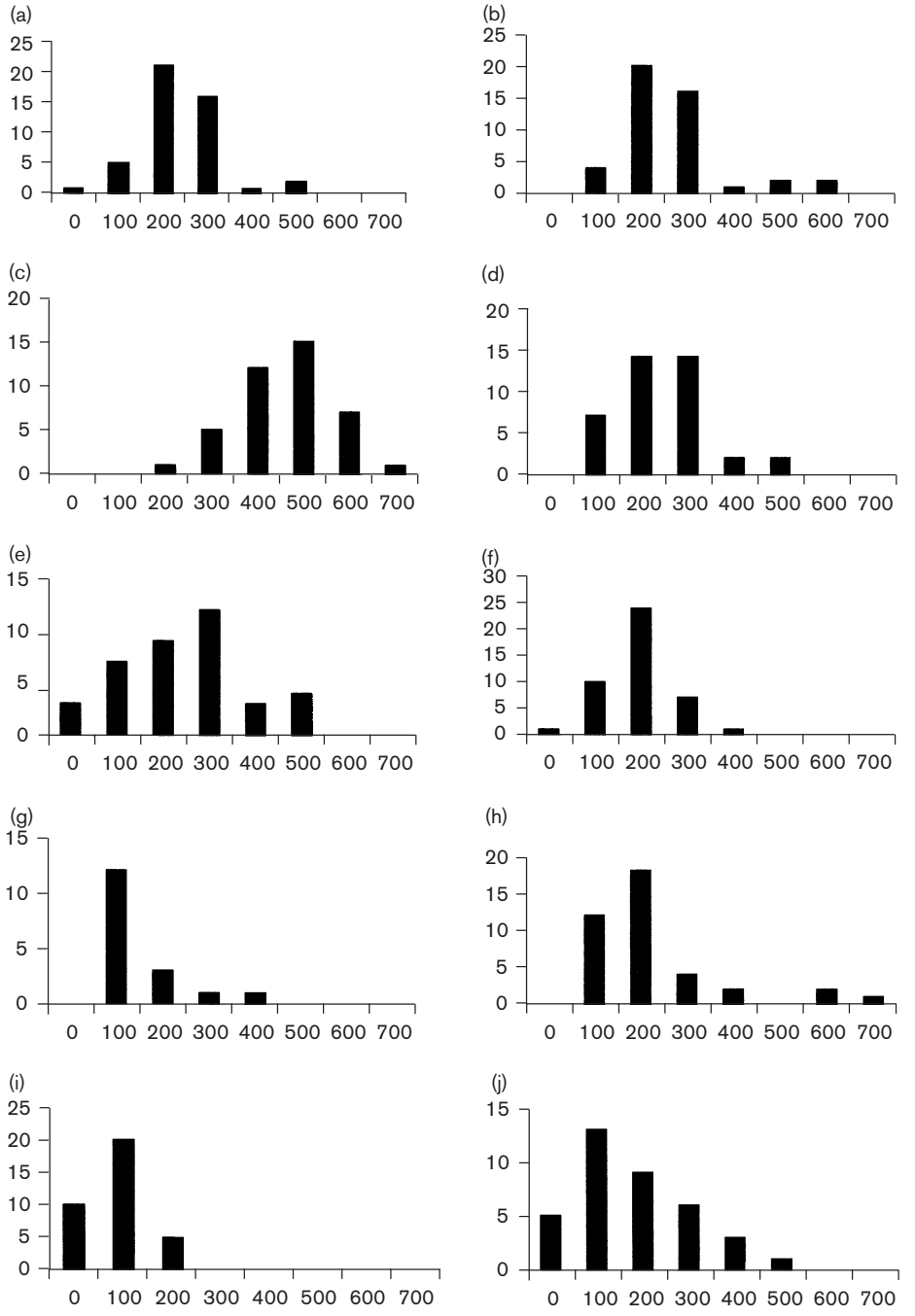


Results for a colorectal ATP-TCA tested (a) in CAM (without additional antibiotics), and (b) with additional amphotericin B (2.5 μg/ml) and metronidazole (1 μg/ml)

Despite appearing sensitive to certain drugs using the $Index_{SUM}$ threshold of < 300, many tumors did not reach 90% inhibition at 100% TDC. Only 15% of tumors tested with 5-FU reached 90% growth inhibition (seven of 46), compared to 0% tested with oxaliplatin (none of 41) and 51% with irinotecan (23 of 45); 90% growth inhibition was achieved in 42% treated with 5-FU + oxaliplatin (18 of 43), 69% with 5-FU + MMC (27 of 39) and 88% with 5-FU + irinotecan (14 of 16).

Nine samples yielded only enough cells for one plate in which we tested the four drugs/combinations most commonly used clinically in the UK. This was due to the fibrotic nature of the samples which made dissociation difficult. The two rectal samples that had undergone neoadjuvant chemoradiotherapy were also small and very fibrotic, resulting in few cells available for testing. Further patients who had already undergone neoadjuvant treatment were excluded from entering our study.

Fig. 2



Frequency histograms showing heterogeneity of the sensitivity index (*y*-axis) for each single agent and combination. (a) 5-FU ($n=46$), (b) irinotecan ($n=45$), (c) oxaliplatin ($n=41$), (d) MMC ($n=39$), (e) gemcitabine ($n=38$), (f) 5-FU + oxaliplatin ($n=43$), (g) 5-FU + irinotecan ($n=17$), (h) MMC + 5-FU ($n=39$), (i) MMC + gemcitabine ($n=35$) and (j) oxaliplatin + gemcitabine ($n=37$).

Discussion

Heterogeneity

The results show the marked heterogeneity of chemosensitivity of CRC to both single agents and combinations of cytotoxic drugs. The proportion of tumors that responded strongly to combinations was greater than the proportion that responded to single agents. The drugs and combinations found effective in this assay are similar to those found to be active in clinical trials, suggesting that the ATP-TCA is able to predict

sensitivity and resistance to chemotherapy in individual patients.

The evaluability of colorectal samples using the ATP-TCA was 87%, which is similar to evaluability rates achieved in other 'cleaner' tumor types using this assay [12,18,19]. Other *in vitro* studies of CRC cells, including the use of the MTT assay and histoculture drug response assay, have produced similar evaluability rates [20,21]. The ATP-TCA has been shown to be more sensitive than these assays, and to have technical advantages over the MTT and clonogenic assays [22,23].

Table 2 Summary of sensitivity data (using an arbitrary threshold of sensitivity defined as a TCA index <300 for six concentrations used)

Drug	No. sensitive	No. in ATP-TCA	Sensitivity assessed (%)
5-FU	27	46	59
Irinotecan	24	45	53
Oxaliplatin	1	41	2
MMC	21	39	54
Gemcitabine	19	38	50
SN38	0	11	0
5-FU + oxaliplatin	36	43	84
5-FU + irinotecan	15	17	88
5-FU + MMC	30	39	77
MMC + gemcitabine	35	35	100
Oxaliplatin + gemcitabine	27	37	73

Infection

The main difficulty in applying primary cell assays in the routine pathology department is microbial contamination. At the commencement of our study there were several plate infections which we overcame by adding additional antibiotics to the assay medium. These antibiotics were shown to have no effect on cytotoxic sensitivity.

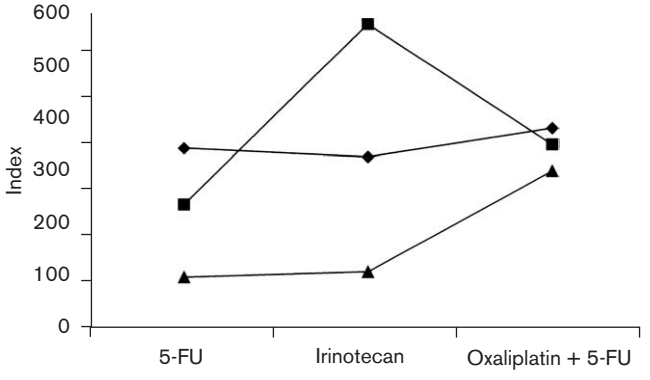
The antifungal amphotericin B was used because the mechanism of action of other antifungal agents renders them unsuitable for use in the assay. Amphotericin B has been shown to reverse resistance and to enhance the cytotoxicity of cisplatin and its analogs *in vitro* [24,25,26]. This is due to an increased intracellular accumulation of drug and an increase in interstrand cross-link formation [27]. Amphotericin B does not seem to have a synergistic effect with 5-FU or a number of other drugs [28,29]. We did not find the addition of amphotericin B in small concentrations (2.5 μg/ml) to have any significant effect on the cytotoxicity of the drugs tested, including cisplatin and oxaliplatin.

Metronidazole is a synthetic antiprotozoal and antibacterial agent. It is known to interact with several drugs, including warfarin, lithium, antiepileptics and cimetidine, as well as alcohol and disulfiram. The only documented interaction with chemotherapeutic agents is with the

Table 3 Median values (and ranges) for each drug and combination

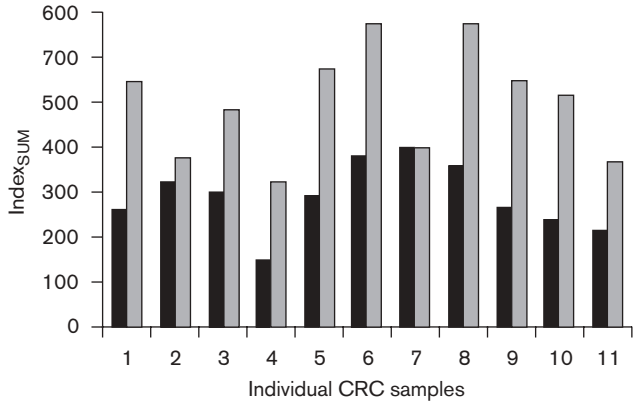
Drug	AUC	IC_{50}	IC_{90}	$Index_{SUM}$
5-FU	13999 (9384–17788)	35 (4–107)	184.5 (45–251)	285.5 (94–528)
Irinotecan	14714 (−931–17885)	37 (5–981)	99 (42–1765)	294 (106–674)
Oxaliplatin	3257 (−2734–12702)	304 (−8104–41192)	546 (−14588–74145)	508 (272–719)
MMC	14049 (4711–18102)	41 (5–223)	173 (45–402)	295 (115–543)
Gemcitabine	11600 (3296–18145)	23 (4–345)	260.5 (31–2500)	298.5 (78–560)
Oxaliplatin + 5-FU	15289 (10440–17910)	23 (5–78)	118 (48–231)	238 (92–403)
Irinotecan + 5-FU	17456 (10876–17879)	11 (6–82)	60 (45–201)	164 (127–414)
MMC + 5-FU	16027 (−10437–18099)	23 (−1223–181)	85 (−2201–304)	235 (104–814)
MMC + gemcitabine	17693 (13762–19277)	7 (3–24)	48 (6–216)	130 (8–268)

Fig. 3



Heterogeneity data for three single agents expressed as TCA indices for three tumors. A low index (<300) indicates probable sensitivity.

Fig. 4



Sensitivity indices for individual tumors tested with irinotecan (solid bars) and SN38 (shaded bars). A low index (<300) indicates probable sensitivity.

alkylating agent melphalan [30]. We did not find low concentrations of metronidazole (1 μg/ml) to alter the chemosensitivity of the drugs tested.

Quality of sample

In general, the colorectal samples were quite fibrous and therefore did not dissociate in the enzymatic solution particularly well. It was necessary to increase the concentration of collagenase from 1 to 1.5 mg/ml for better tumor dissociation. Even so, some tumors did not dissociate well and only yielded enough cells to test a limited set of drugs. Two patients early in the study had undergone preoperative chemoradiotherapy. Irradiation of rectal tumors may produce so much shrinkage that little or no tumor remains macroscopically. We therefore decided not to include any further patients who had undergone radiation therapy in our study.

Drugs

The drug concentrations used in this study are based on their peak plasma concentration (C_{max}), taking into account their degree of protein binding [12]. We acknowledge that this concentration is not necessarily a good indicator of clinically attainable intra-tumor concentrations, but it allows good comparison between agents of different types.

We tested the topoisomerase I inhibitor irinotecan (CPT-11) and its active metabolite SN38 at equivalent doses derived from pharmacokinetic data. Single-agent irinotecan showed activity in 53% of samples tested, but SN38 produced very little growth inhibition in any. Similar results were found by Jonsson *et al.*, who showed irinotecan and SN38 had almost identical activity when tested on cell lines, but that only irinotecan was active when tested *ex vivo* on CRC cells [31]. The inactivity of SN38 suggests that this metabolite of irinotecan may not be relevant and that SN38 is not a good model to demonstrate the activity of irinotecan in tumor-derived cells. It also highlights the importance of using primary cultures of human cells rather than established cell lines, which may show considerable differences in chemosensitivity from tumor-derived cells [32,33].

New regimens

We tested some experimental regimens not currently used in clinical practice (e.g. MMC + gemcitabine and oxaliplatin + gemcitabine). Both these combinations contain gemcitabine which showed some activity as a single agent in 50% of samples. MMC + gemcitabine was the most sensitive combination tested overall. All samples were sensitive to MMC + gemcitabine using the Index < 300 threshold and this was the most sensitive combination in 83% of samples tested.

Gemcitabine has the ability to inhibit DNA replication and repair making it a suitable agent for combination with DNA damaging therapy. The rationale for the combination with MMC is that gemcitabine may enhance the DNA alkylation by MMC or inhibit the repair of its adducts [34,35]. The low toxicity profile of gemcitabine and its clinical activity against numerous solid malignancies make it an attractive drug for use in combination therapy.

The ATP-TCA has been used to assist drug and regimen development [19,36,37]. In colorectal carcinoma, it can be routinely applied to samples obtained from surgical specimens and malignant effusions. The results obtained mirror the heterogeneity of responses observed in clinical studies. Correlation of *ex vivo* ATP-TCA results with clinical response is required to initiate randomized controlled trials of ATP-TCA directed chemotherapy in patients with metastatic CRC.

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