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Heterogeneity in cancer cells: variation in drug response in different primary and secondary colorectal cancer cell lines in vitro

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Abstract Tumor heterogeneity may give rise to differential responses to chemotherapy drugs. Therefore, unraveling tumor heterogeneity has an implication for biomarker discovery and cancer therapeutics. To test this phenomenon, we investigated the differential responses of three secondary colorectal cancer cell lines of different origins (HCT116, HT29, and SW620 cells) and four novel primary cell lines obtained from different colorectal cancer patients to 5-fluorouracil (5-FU) and oxaliplatin (L-OHP) and explored the differences in gene expression among the primary cell lines in response to exposure to cytotoxic drugs. Cells were exposed to different doses of 5-FU and L-OHP separately or in combinations of equitoxic drug or equimolar drug ratios (median effect of Chou-Talalay principle). Cell viability was assessed using MTT assay and the respective IC_{50} values were determined. Changes in gene expression in primary cell lines after exposure to the same drug doses were compared using real-time PCR array. The sensitivities (IC_{50}) of different cell lines, both secondary and primary, to 5-FU and L-OHP were significantly different, whether in monotherapy or combined treatment. Primary cell lines needed higher doses to reach IC_{50}. There were variations in gene expression among the primary cell lines of different chemosensitivities to the challenge of the same combined dose of 5-FU and L-OHP. The results confirm the heterogeneous nature of colorectal cancer cells from different patient tumors. Studies using primary cancer cells established from patient's tumors rather than secondary cell lines will more closely reflect the actual character of the disease.

Keywords Colorectal cancer · Fluorouracil · Oxaliplatin · Drug combination · Chou-Talalay

Introduction

It is now known that many cancers and tumors are not homogenous in nature but instead contain a multiplicity of cells of different characteristics. Tumoral heterogeneity has been well documented in many cancers including colorectal cancer (Raimondi et al. 2014), breast cancer (Geyer et al. 2010), ovarian cancer (Lee et al. 2015), brain cancer (Heppner 1984), and prostate cancer (Boyd et al. 2012). The existence of heterogeneous populations of cancer cells among patients, among tumors, and even within a tumor may lead to different sensitivities to drugs, resistances to treatment, and differences in outcome and relapse after initial treatment. Thus, tumor heterogeneity is recognized as a major hurdle to a cure (Quintana et al. 2010; Burrell et al. 2013; Fisher et al. 2013). Therefore, cellular heterogeneity is likely to be an important factor in determining the overall drug response in cancer treatment.

The elucidation of the human genome and associated advances in molecular biology has enabled tremendous advances in the molecular biology of cancer. Thus, the gene profile of various cancers have been mapped out, and specific mutations or malfunction of various genes have been

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associated with the transformation of normal cells to cancer cells (King and Robins 2006).

The responses and development of resistance of cancer cells to various chemotherapeutic drugs to prolonged drug treatment have been associated with changes in various genes (Housman et al. 2014). The gene profiling of homogeneous secondary cells derived from cancers with known and different resistances to drug treatment and from tumors of patients who have developed resistance to drugs have led to proposals that certain genes or alterations of their functions may be responsible for the development of resistance to continued drug treatment (Sharma et al. 2010; Goodspeed et al. 2016).

Much of the data that have been obtained have come from in vitro studies on various cell lines, particularly homogeneous secondary cells, and it is always problematic to translate these responses to the in vivo situation. It must be realized that tumors are living heterogeneous entities with complex interactions among cells and the sum total of a particular response to drugs is probably dependent on the microenvironment that the cells reside in including cell to cell communications (Edmondson et al. 2014). This notion has gained ground in the last few years and more research is now emerging using heterogeneous primary cells that are derived from patient tumors (Dangles-Marie et al. 2007; Valente et al. 2011). Recently, primary cell banks have been set up for the purpose of drug research and discovery (Mitra et al. 2013).

It has been shown that different patients will respond to different drugs with different sensitivities. Primary cancer cells isolated from the blood of different patients demonstrated differential responses to different drug treatments and has been proposed as a method to provide an initial indication to the most efficacious drug treatment in a particular patient (Greene et al. 2012; Toss et al. 2014). It is an important observation as it means that if a patient could be given the most effective drugs or drug combination at the start of treatment then there is a greater chance of maximal killing of cancer cells as quickly as possible thus reducing the chance of the development of resistance (Friedlander and Fong 2014). However, the main weakness of this approach is that the cells in blood may not mirror the population of heterogeneous cells present in the tumor. Primary cell lines derived from the tumor itself may more closely reflect the actual population of cells present in it.

Drug treatment, dosages, and combinations given are often decided empirically based on evidence of in vitro studies and clinically reported outcomes (Mayer et al. 2006). Thus, knowledge of the initial functional responses of cells to drugs will be useful in determining the most efficacious use of drugs or their combinations to treat a cancer. Low passage primary tumor cell lines more closely resemble the complexity of the original tumor (Ray et al. 2012; Rowehl et al. 2014), including intercellular signaling among heterogeneous subpopulations. As such, these cells could more accurately model in vivo response and ultimately provide insights into the initial design of clinical treatment regimens. We hypothesize that when given similar regimes of drugs, the heterogeneous primary cell lines would show a different cytotoxic response profile than the homogeneous cells line. We also hypothesize that the different cell lines would show differences in resistances to drug treatment.

In this report, we used colorectal cancer cells lines as our study model. We investigated and compared the differences in resistance of three homogenous secondary colorectal cancer cell lines and four low passage primary cells lines derived from four different colorectal cancer patients after exposure with systematically designed equivalent doses of the drugs, 5-fluorouracil (5-FU) and oxaliplatin (L-OHP), either in monotherapy or in equitoxic or equimolar combinations. We analyzed and compared efficacy of the various drugs and possible interactions of these drug combinations in inhibiting cell proliferation, including drug antagonism and synergism. In the primary cell lines, we also explored whether there were changes and differences among these cell lines of different resistance to 5-FU and L-OHP in expression of selected genes including those known to be involved in cell apoptosis, DNA damage repair, and survival.

These data will provide a framework for more detailed studies on the molecular biology and physiology of differential sensitivities of cancer cells, whether among patients, among tumors in the same patient and even within the same tumor to drug response. It is hoped that this will lead to a more targeted and strategically designed modalities for chemotherapy in cancer patients.

Materials and Methods

Drugs The chemotherapeutic drugs, fluorouracil (also known as 5-FU; Hospira, Illinois, USA) and oxaliplatin (also known as L-OHP; Hospira, Illinois, USA), were dissolved and diluted in the appropriate culture media for the experiments.

Secondary colorectal cancer cell lines HT29 colorectal adenocarcinoma (HTB-38) cell line, HCT116 colorectal carcinoma (CCL-247) cell line, and SW620 Dukes' type C colorectal adenocarcinoma (derived from metastatic site: lymph node) (CCL-227) cell line were purchased from the American Type Culture Collection (ATCC, Virginia, USA). The SW620 Dukes' type C colorectal adenocarcinoma cell line was cultured in Dulbecco's Modified Eagle's medium (DMEM), whereas HT29 colorectal adenocarcinoma cell line and HCT116 colorectal carcinoma cell line were cultured in RPMI-1640 medium (Roswell Park Memorial Institute 1640; HyClone, South Logan, Utah, US). Both media were supplemented with 10% of heat-inactivated fetal bovine serum (FBS; Sigma, St. Louis, Missouri, US), 100 units/100 μg of

penicillin/streptomycin (PAA, Linz, Austria), 50 μg/ml of Gentamicin (ATC, Thailand), and 4 mM of L-glutamine (Applichem, Darmstadt, Germany). Cells were cultured in a humidified 5% CO_2 atmosphere at 37 °C based on ATCC cell culture procedure whereby the cell culture media were changed every 2–3 d depending on the cells' growth rate.

Primary colorectal cancer cell lines Four novel primary cell lines were isolated and established from tumor specimens of different colorectal cancer patients based on the protocol described by Arul et al. (2014). The four primary cell lines were established from adenocarcinoma solid tumors (ages 46 to 81 yr), with three (CRC-1, CRC-2, and CRC-3) derived from stage III tumor (or Duke C) and one (CRC-4) derived from stage II tumor (or Duke B). Tumor specimens were first mechanically disaggregated by meticulous mincing, and the disaggregated cells were isolated from tumor biopsy by enzymatic disaggregation via trypsin and collagenase dissociation techniques. First, the minced tissue was immersed in trypsin solution (0.25 wt.%/vol trypsin; Gibco Invitrogen, Carlsbad, California USA) and incubated overnight at 4 °C and then at 37 °C in a humidified 5% CO_2 atmosphere for 30 min. The remaining undigested tissue was immersed in collagenase solution (500 units/ml of collagenase type IV; Gibco Invitrogen, Carlsbad, California USA) and incubated at 37 °C for 1 h. Following dissociation of cancer cells from patient's tumor specimens, the isolated primary cell lines were cultured in RPMI-1640 medium which was supplemented with 10% of heat-inactivated fetal bovine serum, 100 units/100 μg of penicillin/streptomycin, 50 μg/ml of gentamicin, 100 μg/ml of primocin (InvivoGen, San Diego, California, USA), 100 μg of normocin (InvivoGen, US), 4 mM of L-glutamine, 1% of non-essential amino acids (NEAA, HyClone, South Logan, Utah, US), and 1 mM of Na-pyruvate (NEAA, HyClone, South Logan, Utah, US). Primary cell cultures were incubated in a humidified 5% CO_2 atmosphere at 37 °C, and each cell line was maintained at ultra-low passages (i.e., preferably not more than six passages) so that their characteristics would resemble more closely the cells of the primary tumor (tumor collection was obtained with patient's consent and the protocol was approved by University of Malaya Medical Ethics, reference number 589.20).

Cell proliferation assays for secondary cells The cytotoxicity of chemotherapeutic agents was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells in exponential growth were washed with phosphate-buffered saline (PBS), trypsinized with 0.25% trypsin–0.02% EDTA, counted, and seeded in 96-well microtiter plates (TPP, Switzerland) according to predetermined growth characteristics and optimal plating densities for the respective cell lines. HCT116 and HT29 were seeded at a density of 1×10^4 cells/well, and SW620 was seeded at 4×10^3 cells/well. Cells were allowed to attach overnight. Drugs (i.e., 5-FU and L-OHP) in monotherapy or combination were applied in 20-μl volumes to the culture medium. After 72 h of incubation in 5% CO_2 atmosphere and 100% humidity, viable cells were determined using the MTT assay. Twenty microliters of MTT solution (5 mg/ml MTT (Invitrogen, Carlsbad, California USA) was added to each well of the assay plate and then incubated at 37 °C for an additional 3 h. Control wells without drugs were treated identically. After incubation, the culture medium was removed from each well by aspiration. To solubilize the MTT formazan crystals, 150 μl of dimethyl sulfoxide (DMSO; Applichem, Darmstadt, Germany) were added to each well. The plates were left in the dark at room temperature for 10 min, and following this, the plates were gently shaken to enhance dissolution of the crystals. The absorbance values were measured on an ELISA microplate reader (Tecan, Sunrise, Switzerland) at 554 nm with a reference wavelength of 690 nm. Experiments were done in triplicate and repeated three times.

Drug treatment Cells were first exposed to different doses of 5-FU and L-OHP separately or in equitoxic and equimolar (1:1 5-FU/L-OHP molar ratio) combinations. The equitoxic combinations were designed based on the IC_{50} (dose at which half the cells were killed) of the individual 5-FU and L-OHP obtained when drugs were given separately.

(i) Individual drugs (secondary and primary cell lines)

Chemotherapeutic drugs were prepared by diluting in appropriate fresh culture medium according to the required doses. For secondary cells, a serial tenfold dilution of the each cytotoxic drug was prepared in fresh culture medium to give seven descending concentrations of 10,000–0.0001 μM for 5-FU and 100–0.0001 μM for L-OHP. As for primary cells, the individual drug doses consisted of 10, 50, 100, 200, and 400 μM of each cytotoxic drug. The dose-dependent responses were determined, and the IC_{50} for each drug on each cell was estimated.

(ii) Equitoxic drug combination ratios (secondary cell lines only)

Equitoxic dose combinations of these drugs were designed based on the IC_{50} values of each drug. The drug combinations were designed using the Chou-Talalay median-effect principle which uses the constant ratio combination design (Chou and Talalay 1984). This method combines two drugs at a constant equitoxic ratio based on the concentrations of the agents that elicited 50% cell growth inhibition when given separately. Each drug was combined at its equitoxic ratio (i.e., ratio of the IC_{50} concentrations) simultaneously, and then combinations that were two- to fourfold higher and lower than the concentrations at IC_{50}. Thus, five doses of

equitoxic combinations were employed at: 0.25 × IC_{50}, 0.5 × IC_{50}, 1 × IC_{50}, 2 × IC_{50}, and 4 × IC_{50}.

(iii) Fixed equimolar (1:1) drug combination ratios (secondary and primary cell lines)

For secondary cell lines, equimolar drug combinations were combined at 1:1 M ratio with increasing concentrations of 1, 5, 10, 50, and 100 μM. For primary cell lines, the concentrations were 10, 50, 100, 200, and 400 μM. These concentrations used were determined from results of preliminary experiments and were within the clinically relevant ranges: 5-FU (maximum plasma concentration = 426 μM) and L-OHP (maximum plasma concentration = 3.3 mM) (Volkova et al. 2014).

MTT assay was done after 72 h of drug treatment as described in the section above. Control wells received culture media only.

Inhibition of cell proliferation data analysis Results of the MTT cell proliferation assay using single drugs were analyzed using the GraphPad PRISM 5 software (GraphPad Software, Inc.). The IC_{50} values were calculated from the logarithmic trend line of the cytotoxicity graphs. Meanwhile, the combined effects of two drugs in terms of synergism, additive activity, or antagonism were analyzed by the median-effect plot (Chou and Talalay 1984). Drug effects were expressed as percentage inhibition of cell proliferation or fraction of affected cells (Fa) relative to untreated controls (controls were considered to have 0% inhibition or 0 fraction of affected cells, i.e., Fa = 0). Data from three replicates were averaged, and repeats of these data sets (minimum of three) were entered into CalcuSyn (Biosoft, Ferguson, MO, USA) to calculate growth inhibition. The isobologram and combination index methods, derived from the median-effect principle of Chou and Talalay (1984), were used to define the pharmacologic interaction between the chemotherapeutic drugs and cell lines. If the observed effect of two drugs acting simultaneously were larger or smaller than that calculated from the drug expression, it was regarded that synergism or antagonism, respectively, had occurred. The CalcuSyn program uses the median-effect analysis algorithm that produces the combination index (CI) value as a quantitative indicator of the degree of synergy or antagonism. Using this analysis method, CI = 1 reflects additive activity, CI > 1 signifies antagonism, and a CI < 1 indicates synergism (Chou and Talalay 1984).

Genetic expression studies in primary cells Quantitative analysis of changes in selected gene expression in four primary cell lines following a challenge with a fixed equimolar combination dose of 5-FU and L-OHP cells was performed with the customized RT^2 Profiler PCR Array (CAPH13029, PCR Array, Qiagen, Hilden, Germany). In this initial study, 88 genes that were considered to be most relevant in the regulation of apoptosis, cell cycle and proliferation, drug resistance, metabolism and transportation, and various transcription factors were examined. Cells from the four primary cell lines were exposed to a single equimolar-combined dose of 29.3 μM 5-FU and 29.3 μM L-OHP for 72 h. This combined equimolar dose of 29.3 μM of each drug represents the IC_{50} value of the most sensitive cell obtained from preliminary screening of drug cytotoxicity as shown in Table 5. The cell number and culture conditions were as described in the previous section. Control cells (without drugs) received culture media only. After incubation, dead cells were removed using MACS Dead Cell Removal Kit (130-090-101; Miltenyi Biotec, Bergisch Gladbach, Germany) and cells were processed accordingly for genetic analysis. All the in vitro drug-response experiments were repeated three times to ensure reproducibility.

Genetic analysis Genetic expression analysis of control cells and the remaining primary cells after drug treatment were carried out by RT PCR array as described previously by AbuHammad and Zihlif (2013). RNA isolation and purification and cDNA synthesis were performed according to the procedure provided by the manufacturer (Qiagen, Hilden, Germany). The RNA was isolated using an RNeasy Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. An RNase-free DNase set (Qiagen, Hilden, Germany) was used to ensure complete genomic DNA elimination. Complementary DNA strands were synthesized using RT^2 First-Strand kit (Qiagen, Hilden, Germany), and aliquots containing a recommended 0.5 μg of total RNA were used from each sample. Quantitative analysis of gene expression was performed on the customized RT^2 Profiler PCR Array (CAPH13029 PCR Array, Qiagen, Hilden, Germany). In this array, 88 wells contained all the components required for the PCR reaction in addition to a primer for a single gene in each well. The array was customized with selected genes involved in response to chemotherapy and encodes important enzymes that contribute to drug resistance, metabolism, DNA repair, cell cycle, apoptosis, drug transportation, and transcription factors. The PCR array data analysis was performed using the delta cycle threshold (ΔΔCt) method; the analysis was performed automatically according to the SABiosciences Company (Qiagen, Hilden, Germany) web portal (www.SABiosciences.com/pcrarraydataanalysis.php). Changes in gene expression were illustrated as a fold increase or decrease. The data were normalized, across all plates, to the following housekeeping genes: glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*) and 18S ribosomal RNA (*18SrRNA*). The threshold cycle values of the control wells were all within the ranges recommended by the PCR array user manual.

Statistical analysis The statistical significance of differences in drug-treated and control cultures were determined using

one-way ANOVA and post hoc Dunnett's test with the SPSS 14.0 software. Meanwhile, the significance of differences between the IC_{50} values for each cell line was determined using one-way ANOVA and post hoc Tukey's test. The IC_{50} of each drug was determined using the GraphPad software (GraphPad Software Inc., CA). To evaluate whether the interaction between 5-FU and L-OHP was additive or synergistic, the CalcuSyn software (Biosoft, Ferguson, MO) was used to perform isobologram analysis and calculate the CI, according to the Chou and Talalay (1984) method. From the dose-effect plots, the dose that reduced absorbance by 50% (*D*), and the slope (*m*) were calculated. The data were only considered applicable to this method of analysis when the linear correlation coefficient, *r*, of each curve obtained was more than 0.9 (Bijnsdorp et al. 2011). As for gene expression analysis, the *p* values were calculated based on a Student's *t* test of the replicate $2^{(-\Delta\Delta CT)}$ values for each gene in the control group and treatment groups, in which a *p* value less than 0.05 is considered significant. To filter out unreliable data and identify genes with significantly different expression, a standard twofold change in expression was used as an arbitrary cutoff and any fold regulation less than 2 or −2 was considered an unaltered expression.

Results

Inhibitory activity of individual drugs Figure 1 shows that 5-FU and L-OHP when given separately inhibited the three secondary colorectal cancer cell lines in a dose-dependent manner. The estimated IC_{50} of each drug on the three cell lines is shown in Table 1. The three cell lines responded to the drugs with different sensitivities. In terms of the IC_{50} value, HCT116 was most sensitive, followed by HT29 and SW620 was most resistant (Fig. 1*a*). For L-OHP, HCT116 was again most sensitive, followed by SW620 and HT29 (Fig. 1*b*). The IC_{50} values for 5-FU ranged from 1.57 to 92.85 μM among the cell lines (Table 1), while for L-OHP, the IC_{50} values had a narrower range of from 0.58 to 3.81 μM. The IC_{50} values for each drug among the cell lines were significantly different ($p < 0.05$).

Effects of drug combinations in secondary cell lines From the results of the individual drugs, the concentrations for the drugs of the equitoxic drug combination ratio were designed. The base concentrations (in italics) were the IC_{50} of the individual drugs on the three secondary cell lines (Table 2). The other four doses were at 0.25, 0.5, 2, and 4 times the IC_{50} values. The actual concentrations of the equitoxic doses of the drugs used are shown in Table 2 as are the resulting Fa and CI values for each cell line, while those induced by equimolar concentrations of 5-FU and L-OHP are shown in Table 3. Table 4 shows the drug concentrations, given separately, or in equitoxic or equimolar ratios, required to inhibit cell growth to 50% levels, as estimated from the results of the dose-response experiments and Chou-Talalay analysis.

For both equitoxic and equimolar ratios, there were expected dose-response effects of cell inhibition. The concentration of each drug (5-FU and L-OHP) needed to inhibit cell proliferation to the same degree were much lower when drugs were given in combination than when they were given separately (Table 4), which is advantageous. From comparing the data between the equitoxic and equimolar drug treatments, it appears that 5-FU concentrations can be drastically reduced at nearly similar L-OHP concentrations without affecting the efficacy of cell killing. In Table 4, for example, for SW620 cells, the drug concentration that brought about 50% inhibition were L-OHP = 0.91 μM and 5-FU = 44.02 μM in the equitoxic ratio design, whereas in the equimolar design, the same 50% inhibition was achieved with nearly the same L-

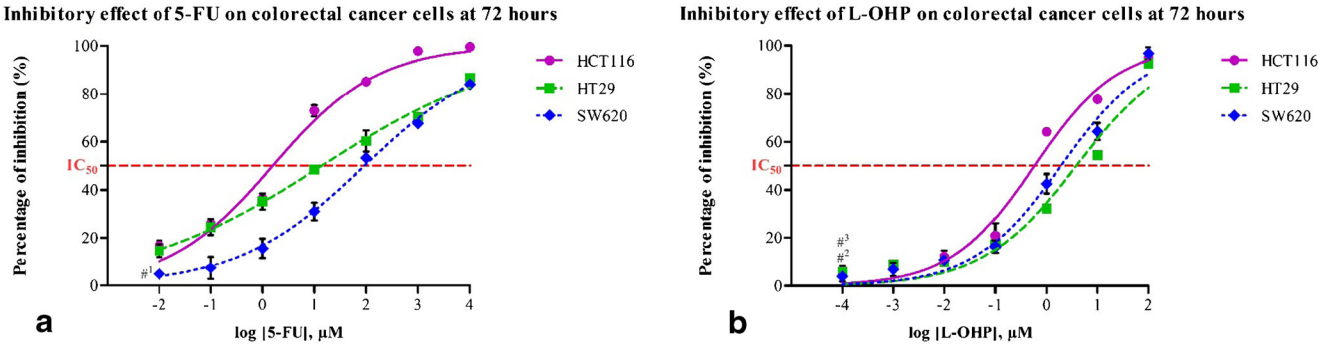

Figure 1. The inhibitory effect of 5-FU and L-OHP on colorectal cancer cell lines. The dose-dependent cytotoxic effect of colorectal cancer cells exposed to 5-FU (a) and L-OHP (b) are shown. Cells were treated with 5-FU and L-OHP alone for 72 h. IC_{50} values (concentration required to reduce the viability of cells by 50% as compared with the control cells) were computed using non-linear regression ($R^2 > 0.95$). Data were expressed as mean ± SEM of three independent experiments (each with samples in triplicates). Values were calculated using GraphPad Prism Software (GraphPad Software Inc.), and the significance of differences in drug-treated and control cultures was determined using one-way ANOVA and post hoc Dunnett's test. All points were statistically significant compared with the control ($p < 0.05$); unless stated otherwise ($^{\#}p > 0.05$, non-significant). $^{\#1}$Non-significant value for SW620 at 0.01 M 5-FU; $^{\#2}$non-significant value for SW620 at 0.0001 μM L-OHP; $^{\#3}$non-significant value for HCT116 at 0.0001 μM L-OHP.

Table 1. IC_{50} values of 5-FU and L-OHP for inhibition of growth of various secondary cell lines

Cell line	IC_{50} (μM)	
	5-FU	L-OHP
HCT116	1.57 ± 0.07*	0.58 ± 0.07*
HT29	13.91 ± 0.06*	3.81 ± 0.09*
SW620	92.85 ± 0.05*	1.91 ± 0.07*

IC_{50} values were calculated using GraphPad Prism Software (GraphPad Software Inc.) from dose-response studies (Fig. 1) and the significance of differences between the IC_{50} values for each cell line was determined using one-way ANOVA and post hoc Tukey's test. Data are presented as mean ± SE

5-FU 5-fluorouracil, *L-OHP* oxaliplatin

*$p < 0.05$ indicates a significant statistical difference in IC_{50} among cell lines

OHP concentrations (1.49 μM) but with greatly lower 5-FU concentrations (1.49 μM). A similar pattern was seen for HT29 cells at 50% inhibition. Therefore, equimolar combination treatment was used for the subsequent drug combination analysis using primary cell lines since an optimum inhibitory effect could be achieved with lower doses in equimolar combinations compared with equitoxic combinations. Furthermore, the CI values were all below 1 for every dose given in equimolar combination compared with equitoxic combination. This indicates a clear synergism in drug action at the levels given.

Drug combination treatment on primary cell lines Primary cells from different patients were first tested with monotherapy of individual drug treatment, and it was found that they inhibited the growth of primary cell in a dose-dependent manner. Each primary cell line displayed different sensitivities to the chemotherapeutic drugs with different IC_{50} values for 5-FU and L-OHP.

For L-OHP treatment, CRC-4 was most sensitive (IC_{50} = 41.7 μM), followed by CRC-2 cells (IC_{50} = 66.2 μM). CRC-3 showed an intermediate sensitivity (IC_{50} = 249.0 μM) while, CRC-1 was considered the most resistant with an IC_{50} of L-OHP at 940.9 μM. The IC_{50} values of L-OHP in all four primary cell lines were within the clinically relevant plasma concentration range for this drug (41.7–940.9 μM).

Meanwhile, for 5-FU, the IC_{50} values covered a wider range (391.9–3091.9 μM) (Table 5). A single dose of 5-FU required to produce a 50% inhibitory effect varied among the cells. CRC-2 was the most sensitive, followed by CRC-4, CRC-1, and CRC-3 cells with significantly different IC_{50} values of 391.9, 1497.0, 2482.6, and 3091.9 μM, respectively.

A reduction of individual drug doses needed to achieve IC_{50} was observed in all primary cell lines when cells were given a combined drug treatment of 5-FU and L-OHP at equimolar ratio (Table 5). In the order of chemosensitivity, the most resistant cell was CRC-1 (IC_{50} reached at a concentration of 258.2 μM of each drug), followed by CRC-3 (IC_{50}, 101.2 μM), CRC-2 (IC_{50}, 38.7 μM), and the most sensitive cell was CRC-4 (IC_{50}, 29.3) (Table 5). Synergism (CI < 1) was evident at the IC_{50} values for all drug combination doses tested on these primary colorectal cancer cell lines.

Gene expression analysis (treated vs. control) The responses in gene expression of selected genes of each cell line to a challenge of a single combined equimolar dose of 29.3 μM 5-FU and 29.3 μM L-OHP each which was equivalent to the IC_{50} for the most sensitive cell line (CRC-4) was analyzed. This dose was chosen as it would kill about half the cells in the most sensitive CRC-4 and CRC-2 cells, with decreasing efficiency in CRC-3 cells and probably leaving essentially all the cells intact in the most resistant CRC-1 cell line. This dose would still leave a sufficiently substantial number of cells intact in the most sensitive cell lines for genetic analysis to facilitate a comparison of the

Table 2. The effects of equitoxic concentrations of 5-FU and L-OHP on Fa and CI of various secondary cell lines

Drug administration (dose)	HCT116			HT29			SW620		
	5-FU/L-OHP concentration (μM)	Fa	CI	5-FU/L-OHP concentration (μM)	Fa	CI	5-FU/L-OHP concentration (μM)	Fa	CI
0.25 × IC_{50}	0.39:0.15	0.36 ± 0.01	1.41	3.48:0.95	0.44 ± 0.002	0.97	23.21:0.48	0.41 ± 0.005	1.21
0.5 × IC_{50}	0.79:0.29	0.45 ± 0.03	1.32	6.96:1.91	0.51 ± 0.006	0.88	46.43:0.96	0.52 ± 0.001	0.90
1 × IC_{50}	*1.57:0.58*	*0.54 ± 0.015*	*1.31*	*13.91:3.81*	*0.58 ± 0.003*	*0.84*	*92.85:1.91*	*0.60 ± 0.013*	*0.84*
2 × IC_{50}	3.14:1.16	0.71 ± 0.016	0.69	27.82:7.62	0.65 ± 0.007	0.77	185.70:3.82	0.67 ± 0.008	0.81
4 × IC_{50}	6.28:2.32	0.80 ± 0.003	0.56	55.64:15.24	0.74 ± 0.008	0.58	371.40:7.64	0.74 ± 0.005	0.78

Equitoxic ratios for testing the drug combinations were chosen on the basis of single drug concentrations that led to a 50% growth inhibition (IC_{50}) in the target cell line (IC_{50}). The base concentrations (set in italics) were at 1 × IC_{50} and the equitoxic ratio of 5-FU and L-OHP were held constant for each cell line as the concentration were increased or decreased. CI values were calculated as a function of the fraction inhibition (Fa) from 0 to 1.0. The CI values represent synergism (CI < 1.0), additivity (CI = 1), and antagonism (CI > 1.0). Data are presented as mean ± SEM

Table 3. The effects of equimolar concentration of 5-FU and L-OHP on Fa and CI of various secondary cell lines

Drug administration 5-FU/L-OHP (μM)	HCT116		HT29		SW620	
	Fa	CI	Fa	CI	Fa	CI
1:1	0.66 ± 0.005	0.62	0.40 ± 0.006	0.96	0.47 ± 0.016	0.67
5:5	0.82 ± 0.008	0.57	0.60 ± 0.006	0.60	0.68 ± 0.003	0.61
10:10	0.88 ± 0.009	0.54	0.68 ± 0.004	0.55	0.76 ± 0.005	0.58
50:50	0.94 ± 0.006	0.52	0.81 ± 0.001	0.54	0.91 ± 0.002	0.32
100:100	0.98 ± 0.010	0.18	0.88 ± 0.001	0.33	0.96 ± 0.004	0.13

The concentration of drugs for fixed ratio combination treatment was in a constant equimolar ratio of 1:1. CI values were calculated as a function of the fraction inhibition (Fa) from 0 to 1.0. The CI values represent synergism (CI < 1.0), additivity (CI = 1) and antagonism (CI > 1.0). Data are presented as mean ± SEM

differences in gene expression that might indicate to the molecular basis for the differences in resistance of the various cell lines to the drugs given.

The genes that showed a significant change in expression across all four primary cells are compiled and compared in Table 6. The cell lines are arranged in the order of drug resistance with the most sensitive (or least resistant) on the left-most column, with increasing resistance and the most resistant cells (i.e., CRC-1 cells on the right-most column). The column to the left of CRC-4 indicates the expected change in resistance if a gene is upregulated: "↑" means an upregulation will increase resistance and "↓" means an upregulation will decrease resistance. The genes grouped in Table 6 summarizes the data for changes in expression of genes (in response to drug treatment) related to apoptosis, DNA repair, drug metabolism, drug transportations, cell cycle control, and various other functions. Only genes showing a significant (twofold) change in expression in at least one cell line are shown. Genes that did not display any significant change in expression in any cell line are not shown.

Of the 88 genes that were examined, 40 genes showed a significant change in expression (i.e., ≥twofold) whether upregulation or downregulation across all the four cell lines. An examination of the changes of gene expression show that the most complex and greatest changes occurred in the most resistant cells (27 genes in CRC-1 cells). Furthermore, not all cell lines had the same changes including those that had quite similar sensitivities (i.e., CRC-4 and CRC-2 cells).

For apoptotic pathway, all cell lines showed increase in expression of various genes that lowered resistance, although the genes were not the same. In the more resistant cell lines, *BCL2*, a gene that confers increased resistance to apoptosis was downregulated. However, in the most resistant cell line (CRC-1), a gene that confers increased resistance to apoptosis (*BCL2L1*) was upregulated.

In genes dealing with DNA repair, the more sensitive cells either had no change (CRC-4) or had more genes downregulated (CRC-2) whereas the more resistant cells had more genes upregulated (CRC-1 and CRC-3).

For genes controlling drug metabolism and various transcription factors, of those investigated, it showed more complex changes in the more resistant cells, both changes that tend to increase or decrease resistance occurred, although in the most resistant cell line, the changes tended to encourage decrease in resistance. As for the transcription factors group, again there were changes in the most resistant cells, with the most resistant cell line showing more changes that would lead to increased resistance (upregulation of *FOS* and *RAF1* and downregulation of *SMAD3*).

Table 4. Drug concentrations needed to inhibit growth of various secondary cells by 50% (determined based on Chou-Talalay method generated by Calcusyn) when given as single drug and in equitoxic or equimolar ratios

Treatment	Growth inhibition	Drug concentration (μM)								
		HCT116			HT29			SW620		
		5-FU	L-OHP	CI	5-FU	L-OHP	CI	5-FU	L-OHP	CI
Single	50%	1.57	0.58		13.91	3.81		92.85	1.91	
Equitoxic combination	50%	0.99	0.37	1.16	6.37	1.75	0.90	44.02	0.91	0.99
Equimolar combination	50%	0.42	0.42	0.96	2.26	2.26	0.73	1.49	1.49	0.78

All values were extracted from dose-response curves, CI and isobologram values which were calculated based on Chou-Talalay mathematical algorithm using Calcusyn software

Table 5. Drug combination treatment versus monotherapy in primary cell lines

Primary cell lines	IC_{50}					
	Monotherapy (μM)		Combination (μM)		CI	Chemoresponses
	5-FU	L-OHP	5-FU	L-OHP		
CRC-1	2482.6	940.9	258.2	258.2	0.38	Resistance
CRC-2	391.9	66.2	38.7	38.7	0.68	Sensitive
CRC-3	3091.9	249.0	101.2	101.2	0.44	Intermediate
CRC-4	1497.0	41.7	29.3	29.3	0.72	Sensitive

Drug doses are significantly reduced in drug combination treatment as compared with monotherapy treatment after 72 h. The equimolar (1:1) drug combination ratios also decreased the drug concentration required to exhibit 50% of cell growth inhibition with synergism observed between drugs (CI < 1). All values were extracted from dose-response curves, CI, and isobologram values which were calculated based on Chou-Talalay mathematical algorithm using Calcusyn software

For transporter genes, the general trend was for the decreased expression of genes that encode for efflux transporters (*ABCB1*, *ABCC1*, *ABCC2*, *ABCG2*, *ATP7A*, and *ATP7B*), which would be expected lead to increased accumulation of drug within the cell and decreased resistance. However, for the most resistant cell (CRC-1) there was an increase in gene expression of the drug efflux transporter (*ATP7B*). In the second most sensitive cell, CRC-2, there was an increase in *SLC22A7* gene which codes for an influx transporter; this would encourage drug accumulation in the cell and thus decrease resistance. In CRC-3, only the expression of one gene was changed and thus the other transport proteins were left intact.

As for the cell cycle control genes the major changes are in upregulation of genes that inhibit cell cycle (e.g., *CKKN1A* and *GADD45A*) or downregulation of genes that allow the cell to proceed (e.g., *CCND1*, *CDK1*, *CCNA1*, and *CCNA2*).

Discussion

This research was undertaken with the premise that cancer tumors even of the same type consist of a multiplicity of cells, which may respond with different sensitivities to chemotherapeutic drugs. The responses of cell to different drugs have been studied extensively, and it is known that different cell lines may respond to and have variations in resistance to drugs (Nannizzi et al. 2010; Mhaidat et al. 2014). In this study, we found that there were significant variances in resistance to drug treatment (5-FU and L-OHP) both in monotherapy and in combined therapy among primary and also secondary colorectal cancer cell lines.

We have also compared the changes in gene expression in four primary cell lines from different patients and found that there were marked differences in gene expression when given the same doses of drug treatment. Although the number of cells examined was limited, these data again reinforce the premise that tumors from different patients are not identical and respond to similar drugs differently. An awareness of the complexities point to the need for more in depth studies of the genes and pathways that confer better treatment strategies in different patients and will further enable a better and rationale initial treatment of cancer, thus enabling a better outcome.

Our experiments using the secondary colorectal cancer cells showed that each cell line had different sensitivities towards 5-FU and L-OHP either in monotherapy or in drug combinations (i.e., equitoxic or equimolar combinations) (Tables 1, 2, 3, and 4). This is probably unsurprising since they are obtained from different sources and may have different degrees of metastatic potential or properties, with SW620 being obtained from metastasized tumor. However, it is still striking that different colorectal cancer cells will have such greatly different responses.

In the secondary cells, we observed that when two drugs interacted synergistically at an equimolar ratio, lower doses of each drug were required to increase the efficiency of drug treatment. This proves advantageous, as the drug concentrations needed to inhibit cell proliferation to the same degree at the higher levels of inhibition were much lower when they were given in combination than when given separately. This is in agreement with Failli et al. (2009), who found that combined treatment with 5-FU and L-OHP was more effective than when drugs are given separately. A number of reports also showed that combined 5-FU and L-OHP treatment in conjunction with another chemotherapeutic drug such as irinotecan, folinic acid and sulindac acid were more effective than 5-FU and L-OHP alone (Fischel et al. 2001; Flis and Splwinski 2009; Toloudi et al. 2015). Clinically, the response rate to monotherapy with 5-FU or L-OHP alone is relatively low at 10–15%, whereas when given in combination, the response rate increased to 40–50% (Douillard et al. 2000; Giacchetti et al. 2000; Johnston and Kaye 2001; Douillard et al. 2003). A synergistic combination of two or more agents

Table 6. Pathway analysis for differentially expressed genes (≥2) in treated primary cells, as compared with the cells in the corresponding control culture (treated vs. control)

Functional gene grouping	Gene symbol	Effect of gene upregulation on drug resistance: ↑ = greater resistance and ↓ = lower resistance	Fold regulation (Sensitive → resistant)				References
			CRC-4	CRC-2	CRC-3	CRC-1	
Apoptosis	BCL2	↑			−3.0	−2.4	*BCL2* and *BCL2L1* are apoptosis-suppressing genes, in which increased expression increases resistance to cell death. All the others are pro-apoptosis genes, whereby, increased expression promotes apoptosis (Manoochehri et al. 2013)
	BCL2L1	↑				5.6	
	TNFRSF10B	↓			2.0		
	TNF	↓		2.6			
	BAD	↓				2.8	
	FASLG	↓		2.8			
	BAX	↓	2.1		2.7		
	FAS	↓			2.9	2.2	
	TNFRSF10A	↓	2.3		3.8		
DNA repair	ERCC2	↑		−2.0			DNA repair genes: increased expression promotes DNA repair and cell survival. (Tomida and Tsuruo 2001; Stoehlmacher et al. 2004; Martin et al. 2008)
	ERCC1	↑		−2.1			
	MSH3	↑		−2.5			
	UNG	↑			2.1		
	ERCC3	↑				2.3	
	ERCC4	↑				3.1	
	XPC	↑		2.2	2.5	3.4	
Drug metabolism	DPYD	↑		−3.0	−2.4	−2.7	Drug metabolism genes: *DYPD* metabolises 5-FU to inactive form; *TYMS* and *DHFR* promote conversion of 5-FU to thymidine which is necessary for DNA repair: upregulation increases resistance. *TYMP* inhibits *TYMS* and *UPP1* metabolizes 5-FU to more toxic form: increased expression decreases resistance (Hato et al. 2012; Kadoyama et al. 2012)
	DHFR	↑	−2.2			−3.4	
	TYMS	↑		2.8	2.8		
	TYMP	↓				2.3	
	UPP1	↓			2.3	5.0	
Drug transporters	ATP7A	↑	−2.0				ATP-binding cassette (ABC) transporters: all except *SLC22A7* are general drug efflux transporters and lowers intracellular drug levels: increased expression increases resistances. *SLC22A7* is an influx transporter (Katano et al. 2003; Kweekel et al. 2005)
	ABCC1	↑		−2.1		−2.8	
	ABCC2	↑	−3.5			−3.9	
	ABCG2	↑		−2.4	−3.8	−5.7	
	ABCB1	↑	−14.3	−3.8		−69.0	
	ATP7B	↑		−2.0		4.3	
	SLC22A7	↓		2.8			
Transcription factors, signal transduction, tumor classification marker, and EMT	ZEB1	↑		−2.5			Various transcription factors affecting growth and apoptosis pathways (Garg and Aggarwal 2002; Orlowski and Baldwin 2002; Altaha et al. 2004; Yang et al. 2006a, b; Gossage and Madhusudan 2007)
	RAF1	↑				2.0	
	FOS	↑	3.9		2.4	5.0	
	SMAD3	↓		−2.5		−3.2	
	NFKBIE	↓			2.2	2.3	
Cell cycle	CCNA2		−2.4	−4.5	−2.7	−15.9	Various cell cycle proteins, difficult to assign resistance with changes in expression (Sherr and Roberts 1999; Smith and Seo 2002; De Angelis et al. 2006; Lin et al. 2006; Yang et al. 2006a, b; Joyce et al. 2011; Agarwal et al. 2013; Christmann and Kaina 2013; Puig-Butille et al. 2014)
	CCNB2		−6.7	−2.0	−4.1	−20.9	
	CDK1		−20.3	−7.4	−5.9	−111.4	
	CCND1		−2.2	−5.0		5.3	
	CDKN2A				2.7	−3.8	
	GADD45A		2.1	3.6	5.3	4.3	
	CDKN1A		2.8	3.0	8.2	6.8	

Changes in expression (≥2) are marked as follows: "positive value"—increase, "negative value"—reduction. Fields left "blank" shows an unaltered expression with a non-significant fold regulation of less than 2

can reduce toxicity and other side effects that are associated with effects of high doses by single drugs. This is may be done by targeting multitarget drug pathway mechanisms (Lehar et al. 2009).

The drug response data from these initial experiments on secondary cells were used to establish the parameters of drug doses and combinations that were to be used for the experiments on the primary cell lines. Four low passage, primary cell lines were established from tumors of four different patients, and therefore these low passage cell lines were unique and were expected to have characteristics that closely resembled the primary tumor cells (Ray et al. 2012; Rowehl et al. 2014). These cell lines, although they were derived from the same cancer type (i.e., colorectal cancer) were very likely to be heterogeneous in nature and different among patients (Marusyk et al. 2012). The responses of these four different cell lines to the drugs proved to be highly interesting. All four primary cell lines exhibited a range of sensitivities to 5-FU and L-OHP when given separately or in equimolar ratios combination as summarized in Table 5. This indicates heterogeneity in cell populations in tumors among patients that may result in different responses to individual drugs (Altschuler and Wu 2010).

Compared with secondary cell lines, primary cell lines required higher doses, whether in monotherapy or in combination treatment, to attain 50% inhibition of cell proliferation. In equimolar ratio combination, all secondary cell lines required less than 5 μM of each drug to achieve IC_{50}, whereas, in primary cells lines, even the most sensitive cells (i.e., CRC-4) needed 29.3 μM of each drug to achieve IC_{50}. This could be due to several factors.

First, secondary cells are immortalized cells that could have transformed away from the characteristics of primary cell from which they originated which could cause changes in sensitivity. Second, primary cells are believed to consist of heterogeneous cell types, each with different sensitivity and thus analysis of drug sensitivity of primary cell lines is probably more complex than a homogeneous secondary cell line. Third, being heterogeneous, there could be cell-to-cell interactions among different cell types, and paracrine secretions from particular cell types may help each other to resist drug treatment.

It is believed that relapse after treatment could also be due to colorectal cancer stem cells (CSC) that may be drug resistant and evade destruction during therapy. CSCs have been shown to be able to exist as adhering and non-adhering cell types in in vitro culture of primary tumors (Dylla et al. 2008; Morata-Tarifa et al. 2016).

Using low adherent culture flasks that discouraged cell adhesion, spheroid colonies derived from dispersed single cells from primary tumors could be grown and transplanted to immunodeficient mice. Mouse xenografts derived from them were enriched in colorectal CSCs, which were shown to be tumorigenic, producing tumors that contain a multiplicity of cells. Cells bearing stem cell markers have also been shown in adherent cell cultures derived from primary CRC tumors including a tumor that has metastasized to the liver (Ray et al. 2012; Rowehl et al. 2014). A comprehensive comparison of drug-responses to CSCs derived from different patients by these methods, both adherent and non-adherent, have yet to be performed.

We have not determined whether there are any stem cells in our low passage heterogeneous cultures. The four cell lines we have obtained have been shown to respond to combinations of 5-FU and L-OHP with different sensitivities and gene expression. If the cells are derived from a single stem cell or specific populations of stem cells, then it is likely that stem cells from different patients may show different response to drug treatment. Thus, more studies will have to be done in this area. It is important to note that CSCs derived from mouse xenografts have been grown in an environment that is foreign to its natural stem cell niche in the colorectal region and divorced from the normal interaction among surrounding cells. Whether this will affect their response to drugs is a matter that has to be considered.

A great deal of information concerning the genetic changes and mutations of various genes and markers characteristic of cancer cells and drug resistance have been obtained (Ku et al. 2010; Ahmed et al. 2013). In our studies, we have focused instead on the changes in gene expression in functional genes due to drug treatment that may be important for resistance, as we are more interested in the physiological processes that confer the differences in responses to drug therapy that we had observed in our cells lines. We have therefore focused primarily on changes in expression of genes directly related to apoptosis, DNA repair, cell cycle, drug metabolism (affecting the intracellular drug levels and thus their effectiveness), and drug transport (primarily efflux transporters which will remove drugs from the cell).

For the genetic expression studies, an equimolar combination dose of 29.3 μM of 5-FU and L-OHP was chosen because it was felt that this would produce the most information with the limited resources available. This dose represented the drug concentration that killed 50% of the population of the most sensitive primary cell line (CRC-4) and also probably CRC-2, which had almost the same sensitivity. This dose would leave considerably more cells intact in CRC-3, which was more resistant, and probably most of the cells intact in the highly resistant CRC-1 cell line. Thus, this dose would leave enough live cells in the most sensitive cell lines to allow a comparison of the genetic expression with the more highly resistant cell lines.

The principle aim of the genetic expression studies was to examine how the different primary cell lines responded to a challenge of similar doses of 5-FU and L-OHP and whether there were any differences in their responses. This would reinforce the evidence that cancer cells from different patients are heterogeneous in nature. Our data showed that indeed the genetic expression change profiles were different in the different cells, even between two cell lines that had relatively

similar IC_{50} values (i.e., CRC-2 and CRC-4). The number of cell lines tested was too few to enable us to draw any relationship between levels of resistance to drugs and the expression of certain genes. A larger number of cells need to be examined and the elucidation of pathways involved lies beyond the resources available to us at present. However, it is clear that there are great complexities and variations in response of individuals to drugs and unraveling the genes and pathways may be an unexpectedly daunting and complicated process.

The data in Table 6 seems to indicate that the more resistant cell lines had more complex changes in gene expression than the more sensitive cells. However, as indicated earlier, the limited data and cell lines tested do not enable us to correlate resistance with changes in expression of specific genes. First, genes from the more susceptible cells in the most sensitive cells might have been lost and thus were not detected. Also it is unlikely that all the genes that may be involved have been captured given the limited number of genes examined.

The result however suggests that no two tumors are alike, and understanding and working out the underlying mechanisms for responses to drugs and drug resistance may be more highly complex than expected. In some instance, changes in gene expression that would increase and reduce resistance sometimes are expressed in unexpected directions. For example, in the apoptosis and DNA repair groups, in the more resistant cells, there was both upregulation in a number of pro-apoptotic genes that would be expected to reduce drug resistance and upregulation in more DNA repair genes, which would be expected to increase resistance. The less resistant cells (i.e., CRC-4 and CRC-2) although possessing about similar sensitivities to the drugs demonstrated striking differences in gene expression; whereby CRC-4 showed no changes in the DNA repair group and upregulation of one gene in the pro-apoptosis group while CRC-2 had a mix of downregulation and upregulation of different pro-apoptosis and DNA repair genes. A review of the other genes also shows a complex mix of up- and downregulation of different genes involved in drug metabolism, drug transportation, transcription factors, and cell cycle control that are different in each cell line. Whether these came from the same cell population or from different cells of a heterogeneous population cannot be determined, and it is not possible at this time to correlate differences in drug resistances to changes in specific genes. It is clear however, that reactions of different cell lines from different patients were not the same, even from two cell lines with approximately the same sensitivities to the drug treatment, i.e., CRC-4 and CRC-2. The ability to resist death from the chemotoxic drugs may well depend from the balance of factors that repair the cell and forces that break down the cell, especially DNA repair and apoptosis.

The events need not come from one cell population, but could come from different subpopulations. A group of cells in the tumor consists of a dynamic living entity with complex interactions among cells. Each individual's genes are different and it is not surprising that each individual's tumors may evolve mechanisms both intracellular and intercellular to resist the abuse of cytotoxic drugs. All these data point to the need for more research to provide a better understanding of drug responses in colorectal cancer.

Conclusions

In conclusion, our experiments confirm that cancer cells, whether homogeneous immortal secondary cells or primary cells from tumors are heterogeneous in nature and respond to drugs differently with different resistances. Comprehensively, we compared the effects of chemotherapeutic drugs 5-FU and L-OHP both in monotherapy and in equitoxic and equimolar combinations first in homogeneous cells and then in low passage primary cells. We showed that lower doses of individual drugs can be used when given in combination than when given separately and at certain combined doses there was synergism. From our results, we can further conclude that homogenous cells are more sensitive to drugs than primary cells whether in monotherapy or combination; thus, relying on drug testing on homogeneous secondary cells alone will overestimate the effectiveness of drugs and lead to treatment doses that are too low to be effective. Testing on primary cells derived from patients will be more reliable. Different patients have different resistances and so testing on primary cells derived from the patients themselves is a better indication of types, doses, and combinations of drugs that are most effective to be used. We showed that primary cells from different patients show differences in gene expression to drug treatment, even from cell lines possessing almost similar resistance to the same drugs. The limited number of genes and cell lines used preclude a correlation of gene expression to drug resistance. However, more extensive studies need to be done before any definite conclusion can be made in this aspect.

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