EXPLORATORY STUDY OF THE ERCC-1 GENE EXPRESSION IN COLORECTAL CANCER CELL LINES AND IN PATIENTS WITH COLORECTAL CANCER

Principal Investigator:

Sanjay Goel, M.D.
1695 Eastchester Road
Montefiore Medical Center Bronx, NY 10461
Phone: 718-405-8404
Fax: 718-405-8433
sgoel@montefiore.org

Co-Investigators:

Andreas Kaubisch, M.D.
Montefiore Medical Center
Hofheimer 100
111 East 210th Street
Bronx NY 10467
Phone: 718-920-4826
Fax: 718-798-7474
akaubisc@montefiore.org

Jennifer Chuy, M.D.
Montefiore Medical Center
1695 Eastchester Road
Bronx, NY 10461
Phone: 718-405-8404
Fax: 718-405-8433

Lakshmi Rajdev, M.D.
Montefiore Medical Center
1695 Eastchester Road
Bronx, NY 10461
Phone: 718-405-8404
Fax: 718-405-8433
lrajdev@montefiore.org

Karenza Alexis, M.D.
Department of Oncology
Jacobi Medical Center
Building 1, Room 3N201400 Pelham Parkway South
Bronx, New York 10461
Phone: 718-918-5753
Fax: 718-918-5578
Karenza.Alexis@nbhn.net

Study Coordinator

Imran Chaudhary, M.D.
Montefiore Medical Park
1695 Eastchester Road, Oncology
Phone 718-405-8542
Fax: 718-405-8527
ichaudha@montefiore.org

Biostatistics:

Abdissa Negassa, Ph.D.
Albert Einstein College of Medicine
Jack and Pearl Resnick Campus
1300 Morris Park Avenue
Belfer Building, Room 1303
Bronx, NY 10461
Tel: 718-430-3575
Email: anegassa@aecom.yu.edu

Participating Institutions
Montefiore Medical Center, Bronx, NY
Jacobi Medical Center, Bronx, NY
Albert Einstein College of Medicine of Yeshiva University, Bronx, NY

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1 OBJECTIVES

1. **Determine extent of up regulation in the ERCC-1 gene expression on exposure to oxaliplatin *in vitro* and *in vivo*.**

1a. The magnitude of ERCC1 up regulation in response to oxaliplatin treatment will be correlated with apoptotic and growth inhibitory response in a panel a 30 colon cancer cell lines previously screened for response to this agent in the Montefiore laboratory.

1b. Directly test the link between up regulation of ERCC-1 and oxaliplatin response. ERCC-1 gene expression will be silenced in oxaliplatin responsive colon cancer cell lines using siRNA, and response to oxaliplatin determined.

1c. Determine whether changes in ERCC-1 gene expression occur *in vivo* in patients treated with oxaliplatin, and to determine whether the magnitude of ERCC-1 induction correlates with patient response. In addition, whether basal ERCC-1 expression is a determinant of oxaliplatin response will also be determined.

1d. Determine whether changes in ERCC-1 gene expression can be detected in the peripheral blood mononuclear cells (PBMC) on exposure to oxaliplatin, and to determine a correlation coefficient between the magnitude of change in gene expression in the colorectal cancer specimens and the PBMC.

2 **Determine the ERCC-1 C→T polymorphism and determine the association between the presence of polymorphism and resistance to oxaliplatin.**

2a. The link between ERCC-1 polymorphism and magnitude of ERCC-1 induction in response to oxaliplatin will also be determined.

2b. Determine whether a specific ERCC-1 polymorphism is linked to objective response to oxaliplatin *in vivo*, and further, to determine whether a specific polymorphism is linked to the magnitude of ERCC-1 induction in response to oxaliplatin.

3 **Establish a gene expression pattern that will predict response to oxaliplatin in colorectal cancer cell lines.**

3a. This will identify multigene markers as opposed to a single genetic marker.

3b. Identify genes altered in expression in response to oxaliplatin treatment. The fresh tumor samples will be screened using the array technique.

2.0 BACKGROUND AND RATIONALE

Colorectal cancer is the second leading cause of cancer death among both men and women, accounting for over 56,000 deaths annually [2]. For patients with advanced colon cancer, oxaliplatin (trans-L-1, 2-diamino cyclohexane oxalatoplatinum) has been approved for therapy as a front line agent [3]. The platinum drugs include the prototype cisplatin, and its analogs, carboplatin and oxaliplatin, and have been the mainstay of therapy for lung, ovarian, breast, cervical, head and neck, and testicular cancer. These drugs exert their anti cancer activity by forming inter-, and intra- (DNA) strand platinum adducts thereby causing DNA damage and

inhibiting cell replication. In spite of these advances, the benefits that patients derive remain dismal with response rates in patients with advanced non-small cell lung cancer in the range of 15-20%, and median survival of 9 months at best [4]. For patients with metastatic colon cancer, with the incorporation of new agents, including oxaliplatin, the median survival has improved to 24 months with response rates of 50-60% [5].

DNA damaging agents depend on irreversibly damaging the cell's DNA in order to exert an anti cancer effect. Successful anti cancer therapy is limited by the cancer cell's ability to bypass the lethal action and subsequent development of drug resistance. In the case of the platinum compounds, the resistance of the cancer cell to the chemotherapy is multifactorial [6,7,8] and includes: reduction in drug entry and subsequent accumulation within the cell [6,8]; enhanced detoxification by the glutathione and metallothionein systems by sequestration of platinum and prevention of formation of DNA adducts [6,9,10,11]; altered patterns of platinum-DNA adduct formation [12,13]; prevention of DNA damage after the formation of the DNA-platinum adducts by either the ability to rapidly repair the DNA damage [3,4,5,11], or by development of tolerance to the DNA damage [12,13]. The repair of DNA damage is made possible by the nucleotide excision repair (NER) pathway. Two major genes involved in this pathway are the excision repair cross-complementation group 1 (ERCC-1) and the ERCC-2 (xeroderma pigmentosa - XPD) gene. It has been well established that expression levels of the ERCC-1 gene can significantly affect the ability of the drug to influence survival in patients with colon cancer [14]. The NER reaction is carried out by a multienzyme complex and undergoes a stepwise process of recognition, incision, excision, repair synthesis and ligation [15,16]. The ERCC-1 protein forms a complex with the XPF product, which leads to development of endonuclease activity [15]. This then excises DNA-platinum adduct at the 5' end of the damaged strand [17,18]. Thus far, NER is the only known mechanism for the removal and subsequent repair of the damage produced by the platinum drugs [19].

Studies on ovarian and colon cancer cell lines have demonstrated that there is up regulation of gene transcription with a rise in both mRNA and protein levels on exposure to cisplatin and oxaliplatin, respectively. We therefore hypothesize that up regulation of ERCC gene expression in colorectal cancer cell lines is a reason for the different sensitivity patterns among the 30 colon cancer cell lines. Studies have demonstrated that the extent of DNA damage repair as evidenced by the ERCC-1 expression levels is 30-50 times higher in resistant cells as compared to hypersensitive cells [20]. Other studies have also proven that in addition to colon cancer, the expression of the ERCC-1 gene also predicts for survival and cisplatin resistance in patients with lung [21], gastric [22] and ovarian [23] cancer. Enhanced ERCC expression is directly linked to clinical response to cisplatin in ovarian cancer patients [23,24]. It has been clearly established that cells that are defective in this pathway are hypersensitive to the platinum [25,26], and those overactive in this pathway are resistant [8,27,28]. In a study involving 4 cancer cell lines, ovarian and an inherently cisplatin resistant colon (HT-29) cell lines were used. ERCC-1 mRNA levels were measured after exposure to oxaliplatin for 20 hours and were higher than in the control, the A2780 (ovarian) cell line [29]. Another group studied the combination of irinotecan and oxaliplatin in HCT-8 cell lines and xenograft modes and observed that the ERCC-1 expression was upregulated on exposure to oxaliplatin. Addition of irinotecan abrogated this effect, with the potential for synergy between the two drugs by the inhibition of DNA repair and increased cytotoxicity of the platinum [30].

Investigating ERCC-1 gene polymorphisms, DNA was collected from a clinical study evaluating the use of oxaliplatin in patients with advanced colorectal cancer and the C→T single nucleotide polymorphism in exon 118 was studied. Patients with the C/C genotype had the most favorable survival. The homozygous TT and the heterozygous CT patients had a relative risk of dying of

1.86 and 2.29 (p=0.021); respectively [31]; however there is no evidence that the polymorphism bears an association with response. Similar findings have been observed in lung cancer [32,33]. In a group of cisplatin treated patients, the C/C homozygous genotype was associated with a median survival of 486 days and was significantly (p=0.0058) greater than the T/T genotype with a median survival of 281 days. We also propose to look at polymorphisms in these cell lines and establish the presence or absence of an association between the genetic polymorphisms, and the cellular sensitivity to the cytotoxicity of oxaliplatin.

While there is extensive pre-clinical data suggesting the influence of ERCC-1 on platinum sensitivity, *in vivo* data continues to be lacking. The aim of this proposal is to study changes in ERCC-1 gene expression in PBMC and fresh tumor tissue, both before and after oxaliplatin therapy. While it is critical to determine the expression pattern within the malignant cell, practically, it is difficult to obtain fresh tumor specimens, especially if one is interested in serial sampling to study changes in RNA over time, or on exposure to drugs or toxins. Some studies have demonstrated that in certain circumstances, peripheral blood mononuclear cells (PBMC) could serve as a reliable surrogate marker for cancer tissue. Smoking induced carcinogen-DNA adducts were compared and levels from PBMC were found to be a reliable surrogate for levels in lung cancer specimens [34]. Patients with renal cancer were treated with the mTOR inhibitor CCI-779, and the gene expression profiles from their PBMC reliably identified the expression profile with a higher likelihood of a response [35]. Another mTOR inhibitor RAD 0001, was looked at in a syngeneic rat pancreatic tumor model, and it was found that long term monitoring of PBMC derived S6K1 activity levels could be used for assessing RAD 001 treatment schedules in patients with cancer [36]. The PBMC may reflect the events in the cancer cell and it is important to make this determination and derive a relationship between the gene expressions in the two sources of cells. The study will look at ERCC-1 gene expression in PBMC and determine a correlation between the changes in the expression levels in the PBMC and fresh tumor tissue. If it can be established that the PBMC serves as a surrogate marker for the events within the tumor cell, in the future all studies can be readily performed on the peripheral cells with confidence.

Multiple reports have indicated that among patients with colorectal cancer, the survival of African Americans is lower than that for Caucasians. Possible explanations for this disparity is lower socio-economic status, lack of screening, delayed diagnoses, and genetic differences that possibly render one population to derive less benefit from the available therapy [37,38,39]. Retrospective data suggests that baseline ERCC-1 expression and the polymorphisms do impact on survival of colorectal cancer patients who are treated with oxaliplatin. We therefore hypothesize that one factor accounting for the racial disparity in survival is attributable to differences in baseline expression or to a more frequent presence of polymorphism that is associated with a poorer survival.

With the advent of modern micro array technology, it is possible to screen a cell line or tumor sample for thousands of genes at one time and using this technique, research groups have established specific gene expression profiles that predict for sensitivity to cytotoxic drugs [40]. The laboratory of Drs. Leonard Augenlicht and John Mariadason has identified the gene expression profile in a panel of 30 colon cancer cell lines and that could determine sensitivity and resistance patterns to the cytotoxic effects of 5-fluorouracil and irinotecan [41]. They have found that the selection of 50 genes correlated with 5-FU responsiveness, but not a random set of 50 genes. Also found was that this gene expression was a better predictor of 5-FU response than the previous well-established predictors of response, namely, thymidylate synthase, thymidine phosphorylase, p53 and mismatch repair status.

3.0 PRELIMINARY DATA

The cell biology department at the Albert Einstein Cancer Center has studied a panel of 30 colon cancer cell lines and determined a sensitivity pattern to various drugs used in the clinic as a therapeutic option for patients with advanced disease [55]. The laboratory of Dr. John Mariadason has studied a panel of 30 colon cancer cell lines and observed that there is a distinct susceptibility pattern with cells ranging from being highly susceptible (GI 50 of 0.5, 95% apoptosis) to highly resistant (GI 50 of 11, 10% apoptosis) to oxaliplatin [54]. This pattern is hereby displayed below [fig 1]. As is evident, cell lines have been arranged in order of increasing sensitivity to oxaliplatin from least sensitive to the most sensitive. It is this cell lines model that will be used for this project to determine ERCC-1 gene expression profiling, to carry out the gene silencing experiments, and to determine germline polymorphisms.

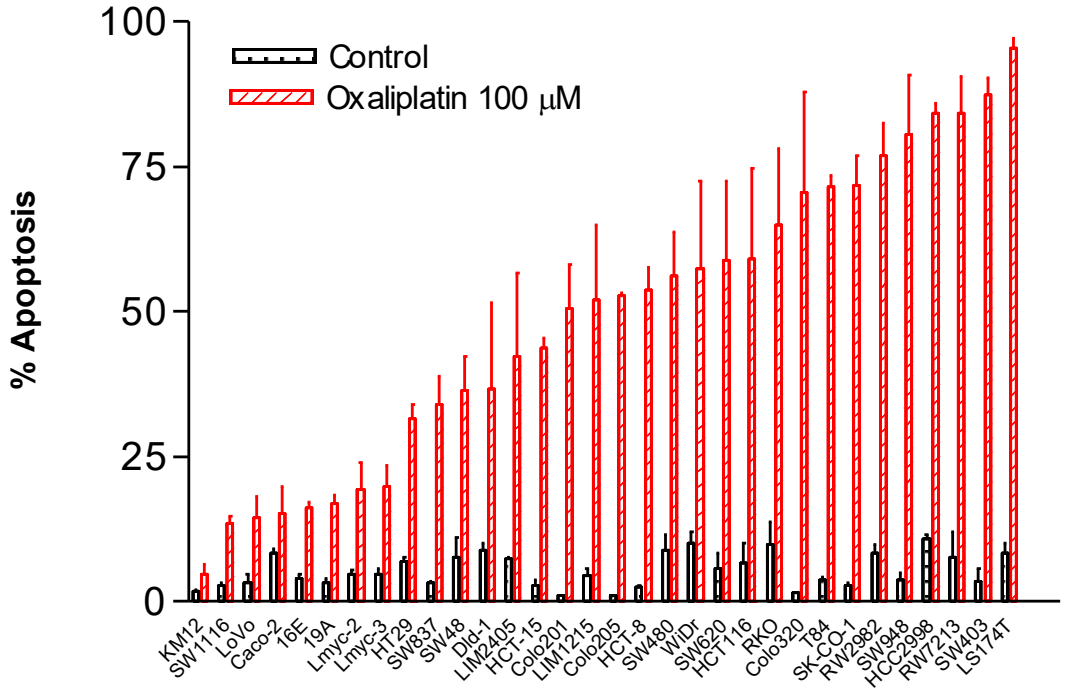
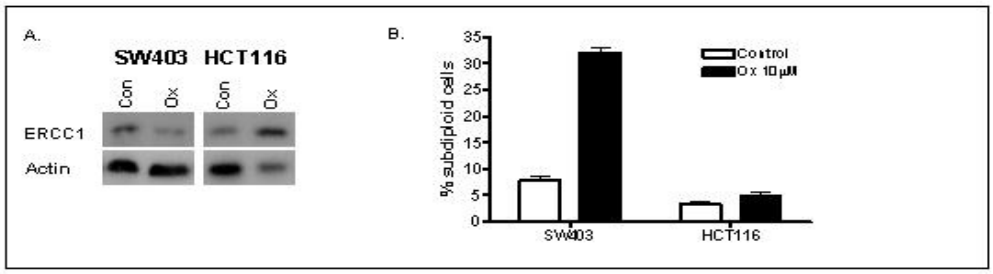


Fig 1 Apoptotic susceptibility pattern of a panel of 30 colon cancer lines to oxaliplatin



Oxaliplatin treatment results in differential ERCC1 induction in colon cancer cells.
Two colon cancer cell lines were treated with 10 µM oxaliplatin for 24 hours, and ERCC1 levels determined by western blot.
Loading was controlled for by reprobing for β-actin. A decrease in ERCC1 was observed in SW403 cells, and an increase in HCT116 cells.

Fig 2: Effect on ERCC-1 gene expression on exposure to oxaliplatin in two colon cancer cell lines

Further, two cell lines were randomly selected and studied for the alteration in the ERCC-1 gene expression after exposure to oxaliplatin at 10 µM for 24 hours. As is evident from this graph, an increase in ERCC-1 expression was observed in the HCT116 cell line while a decrease was observed in the SW403 cell lines.

4.0 PATIENT ELIGIBILITY CRITERIA

Inclusion Criteria

3.1 Histologically confirmed diagnosis of colorectal cancer

3.2 ECOG Performance Status 0-2 (Appendix A)

3.3 Patient is a candidate for oxaliplatin based therapy either in the adjuvant or in the metastatic setting

3.4 Consent to donate 9 tubes of PBMC of 7 ml of blood each

3.5 Willing to consider additional post therapy tumor biopsy if applicable (refusal to consent is not an exclusion criteria)

3.6 Adequate organ function as defined as

Neutrophil count > 1500/µl
Platelets > 75,000/ µl
Hemoglobin > 8 g/dl
Bilirubin < 2.0 X upper limit of normal
Creatinine $\leq$ 2 mg% or calculated clearance $\geq$ 40 ml/mt

3.7 The patient must have signed a consent form approved by the Albert Einstein College of Medicine Cancer Center CCI and Montefiore Medical Center IRB

Exclusion Criteria

3.8 No other significant underlying medical condition that will, in the opinion of the principal investigator or designees, make administration of oxaliplatin unusually hazardous, such as significant hepatic, bone marrow and/or cardiac disease, requiring active medical treatment.

3.9 Pregnant women or women of child bearing potential not practicing birth control or sexually active males unwilling to practice contraception during the study.

3.10 Patients undergoing major surgical procedures (they will be delayed enrollment until complete recovery from their surgery - 4 wk for major or 2 wk from minor surgery).

3.11 Patients with grade 2 neuropathy will not be eligible for the study

3.12 The patient must not have received chemotherapy within 4 weeks of beginning oxaliplatin treatment. At least 6 weeks must elapse if prior therapy included mitomycin C or nitrosoureas. At least 2 weeks must have elapsed since the end of prior palliative radiation therapy.

5.0 REGISTRATION PROCEDURE AND DATA SUBMISSION

All patients will be registered with Milagros Rodriquez in the Cancer Clinical Trials Data Management Office of the Montefiore-Einstein Center for Cancer Care
1521 Jarret Place
Bronx, NY 10461
Phone (718) 379-6861
Fax (718) 822-0335

6.0 TREATMENT PLAN AND STUDY DESIGN

The 30 colon cancer cell lines panel that has been described in the preliminary data section of the protocol will be used. Total ERCC-1 mRNA will be extracted using the RNeasy RNA extraction kit from Qiagen and converted to cDNA using an anchored oligo dT primer and reverse transcriptase. Quantitative real time PCR will be used to quantify ERCC1 mRNA and results expressed relative to GAPDH levels. Total cellular protein will be extracted and ERCC-1 protein level quantified by Western Blotting using an anti ERCC-1 antibody.

Forty patients (20 patients/year) will be enrolled to collect four tubes of 7 ml of blood for PBMC analysis. This will be used to collect cells for ERCC expression. mRNA will be measured by quantitative PCR [22,44] and protein by Western Blotting [45]. This tube of blood will be drawn before commencement of any chemotherapy (0 hours/baseline), at the end of infusion (2 hours), and at 48 hours (this is typically when patients return to the infusion unit to discontinue the 5-FU infusional pump). Another tube will be collected when the patient returns on the first day of the next cycle. The PBMC samples will be processed within 60 minutes of collection.

In addition, patients will be identified before a diagnostic procedure such as an endoscopy, or a CT guided biopsy. The patients will be asked to sign a consent form before the diagnostic procedure that will allow the use their biopsy or resection specimens as a source of tissue. The gastroenterologist performing the endoscopies will biopsy two specimens, of which one specimen will be formalin fixed and sent to pathology for the establishment of a diagnosis. The second will be handed to me my designee and this will be immediately placed in dry ice in the operating/procedure room. The sample will be transported out of the procedure room immediately and then placed in liquid nitrogen. This tissue will be studied for ERCC-1 mRNA expression. For patients from whom we will be unable to obtain fresh tumor samples, only the formalin fixed, paraffin embedded tissue block will be used to estimate ERCC-1 protein content in a semi-quantitative manner using the immune-histochemistry (IHC) analysis using a commercially available antibody to the protein [42]. The IHC staining will be performed using antibodies from biomeda, and analyses using standard techniques described previously [43]. Appropriate positive and negative controls will be used. The IHC will be graded as positive or negative and will be independently verified by a pathologist blinded to the identity of the sample. It is estimated that close to 22%, of patients with unresectable metastatic colorectal cancer are able to undergo curative resection of the metastases after therapy with the FOLFOX regimen [5]. The surgeon performing the liver resection will hand over a piece of the resected specimen to my designee or me and this will be handled in a manner described previously. This will facilitate gene expression study over time after a 2-hour exposure to oxaliplatin. Both, the fresh tissue and the PBMC will be analyzed for semi-quantitative ERCC-1 by IHC staining, and by quantitative mRNA and protein analyses. Analyses to estimate the degree of correlation between the magnitude of change in the ERCC-1 gene expression in PBMC and the fresh tumor samples will be performed.

All patients will undergo serial radiological testing (CT scans) every 2 cycles (each cycle consisting of 4 weeks of therapy in case of FOLFOX or 3 weeks in case of XELOX). The patients will be evaluated for response using the response evaluation criteria in solid tumors (RECIST) as described previously [46].

Silencing of ERCC-1

To directly determine the role of ERCC-1 in mediating cellular response to oxaliplatin in colon cancer cells, we will transfect 2 oxaliplatin-resistant cell lines with siRNAs that specifically target ERCC-1. siRNAs will be purchased from Dharmacon, and transfected into cell lines using lipofectamine 2000 at a final siRNA concentration of 100 nM. As a control, cells will also be transfected with a non-targeting siRNA. The silencing efficiency of the siRNAs will be validated 24 hours post transfection by their ability to inhibit basal and oxaliplatin-induced ERCC-1 expression at the mRNA (QPCR) and protein level (western blot). Once silencing efficiency has been confirmed, apoptotic response of ERCC-1-silenced colon cancer cells treated with 1, 10 and 100 μM oxaliplatin for 72 hours will be compared to cells transfected with non-targeting siRNA. Apoptosis will be assessed by propidium iodide staining followed by FACS analysis. Experiments will be performed in triplicate on 3 independent occasions. In addition to apoptosis assays, we will further test the role of ERCC-1 silencing on oxaliplatin response by clonogenic assay. Cells transfected with ERCC-1 or non-targeting siRNA will be treated with 1, 10 or 100 mM oxaliplatin for 9 hours, following which time the drug will be removed, cells trypsinized, and reseeded in fresh medium at a density of 500 cells per well. Colony formation will be monitored over the following 2 weeks and visualized by staining with crystal violet.

From the panel of 30 colon cancer cell lines, genomic DNA will be extracted and studied for germline polymorphisms as described below. Similarly, from the baseline PBMC from all patients, DNA will be extracted to determine germline polymorphisms. The genomic DNA will be prepared from PBMC using the Puregene blood DNA kit (Gentra Inc.) following the manufacturer's protocol. The ERCC-1 C→T polymorphism will be detected using modified PCR-RFLP methods, using primer sequences described previously [33]. It is well established that certain germline polymorphisms in the ERCC-1 gene predicts for increased survival, however, no such association has been established with clinical response. This proposal will determine an association between the presence of a particular polymorphism with changes in ERCC gene expression, and of the ERCC-1 gene polymorphisms with clinical response.

To gain further insights into the mechanisms of oxaliplatin response and to identify novel molecular determinants for differential cellular response to this agent, we will undertake a non-hypothesis driven microarray-based screening strategy. Based on our preliminary data, we will select the 5 most oxaliplatin-sensitive (LS174T, SW403, RW7213, HCC2998, SW948) and 5 most oxaliplatin-resistant cell lines (KM12, SW1116, LoVo, Caco-2, 16E) for further analysis (10 cell lines in total). Each of these cell lines will be treated with 10 μM oxaliplatin for 24 hours. Total RNA will be isolated from treated and untreated cells using the RNeasy kit (Qiagen, Valencia, CA). For microarray hybridizations, 50 μg of RNA from oxaliplatin treated cells will be labeled with Cy5 dUTP, and 50 μg of RNA from untreated cells labeled with Cy3 dUTP. Probe preparation, hybridization conditions, and array scanning procedure were as previously described and as routinely performed in our laboratory [47,48]. Labeled probes will be hybridized to 27,000 feature cDNA microarrays generated by the Albert Einstein College of Medicine microarray facility [49]. Images will be analyzed using Genepix Pro software (Axon Instruments, Union City, CA), and lowess normalized. For each cell line, two independent oxaliplatin treatment experiments will be performed, and the mean value (ratio) for each gene computed.

Genes differentially expressed between oxaliplatin sensitive and resistance cell lines will be determined using a students t test, with a P value of <0.05 considered to be statistically significant. To reduce the number of false positive discoveries, the mean value across the 5 sensitive and 5 resistant cell lines will be computed, and genes that are differently expressed 2-fold or greater between sensitive and resistant cell lines determined. Genes that satisfy both of these criteria will be considered differentially expressed between oxaliplatin sensitive and resistant cell lines. To gain mechanistic insights into differentially expressed gene lists, we routinely perform enrichment analyses to identify functionally related categories of genes enriched for differential expression. For this analysis we will take advantage of our prior classification of all genes on the AECOM cDNA microarrays into 150 biologically related categories, including cell cycle progression mediators, apoptosis mediators, transcription factors and signaling mediators. We will utilize a Fisher exact test to identify biologically related categories of genes enriched for altered expression in response to oxaliplatin treatment and differentially expressed between oxaliplatin sensitive and resistant cell lines. Differentially expressed genes will be validated by RT-PCR and whether specific candidate genes play a direct role in oxaliplatin response tested by siRNA-mediated silencing experiments followed by reassessment of oxaliplatin response.

The same biopsy material described in aim 1 that will be obtained from patients pre- and post-oxaliplatin treatment will be used to determine the changes in gene expression induced by this agent in vivo. RNA will be extracted from biopsies by homogenization of biopsy tissue in Trizol followed by further purification using an RNeasy column (Qiagen). From previous studies we envision obtaining between 2-5 micrograms of total RNA from patient biopsies [50]. RNA will be linearly amplified by T7 bacteriophage RNA polymerase driven in vitro transcription (Arcturus, KIT 0201). For colon cancer cell lines, this procedure typically yields approximately 30 µg of polyU mRNA from 5 µg of total starting RNA. 5 µg of polyU/polyA mRNA from pre and post treatment specimens will be labeled with Cy5 dUTP and compared to 5 µg of Cy3 dUTP-labeled reference RNA routinely utilized in the Augenlicht laboratory [55]. Comparison of the pre and post treatment samples to a reference RNA will enable additional analyses to be performed in the future, including the identification of genes / signatures whose basal level of expression may be predictive of oxaliplatin response. Patient with good and bad response to oxaliplatin will be separated and genes differentially expressed in response to oxaliplatin identified as described in previously. In addition, genes altered in expression in response to oxaliplatin treatment in vitro and in vivo will be identified.

Oxaliplatin and other chemotherapy will be delivered as is standard of care. However, doses may be modified per treating physician and patient tolerance

FOLFOX (q 14 days)
Oxaliplatin at 85 mg/m^2 over 2 hours on day 1
5-FU at 400 mg/m^2 bolus on day 1
Leucovorin 400 mg/m^2 over 2 hours on day 1
5-FU at 2400 mg/m^2 over 46 hours via infusion pump
Bevacizumab 5 mg/kg on day 1

XELOX (q 21 days)
Oxaliplatin 130 mg/m^2 over 2 hours on day 1
Capecitabine 1000 mg/m^2 q 12 hours on days 1-14
Bevacizumab 7.5 mg/kg on day 1

7.0 SUPPORTIVE THERAPY GUIDELINES

Use of supportive therapy including antiemetic therapy will be at the discretion of the treating physician. However, it is suggested that patients receive a 5-HT3 antagonist, decadron and lorazepam prior to oxaliplatin dosing.
Use of colony stimulating factors (such as Procrit, Aranesp, Neupogen, Neulasta) is allowed and is at the discretion of the treating physician.
For patients with bony metastases, use of bisphosphanate therapy is allowed and is at the discretion of the treating physician.

8.0 MONITORING OF PATIENTS

Patients will be evaluated as is standard of care for a patient receiving oxaliplatin based therapy either alone or in combination with 5-FU (FOLFOX) or with capecitabine (Xeloda® - XELOX). It is suggested that they at least be evaluated once every other week by the treating physician.

All patients will have imaging studies for tumor evaluation at baseline and at the end of two cycles (each cycle consisting of 4 weeks with FOLFOX or 3 weeks of XELOX).

For details, refer to study calender.

9.0 STUDY CALENDAR

All radiological tests and disease assessments have to be within 4 weeks of starting treatment

The consent date is within four weeks of registration for study

All blood tests and physical exam have to be done within 2 weeks of starting treatment

	Pre-study		D 1	D 3	D15	q 2 wk (q 3 wk)[d]	q 3 wk (q 4 wk) [d]	wk 8
	(Day -28 to start)	(Day -14 to start)						
Informed consent	X							
History and Physical		X					X	
Vital Signs		X					X	
Weight		X					X	
Height		X						
BSA		X					X	
PS		X					X	
AE/SAE Monitoring			X				X	
CT scans [a]	X							X
Tumor Measurements [a]	X							
CBC		X			X	X	X	
Chemistry-7		X						
LFT		X						
Beta-hCG [b]		X						
CEA		X					X	
Oxaliplatin dosing			X		X	X		
PBMC collection [c]			X	X	X			

Index for Study Calender

X[a] If a patient has a clearly defined palpable mass or on an X-ray, a CT may be avoided After every two cycles (4 doses)of therapy

X[b] In women of childbearing potential (can be serum or urine).

X[c] PBMC will be collected at baseline, 2 hr (EOI), 48 hr and at D 15

X[d] Bracketed time interval is for XELOX while non bracketed is for FOLFOX

10.0 DRUG FORMULATION

Oxaliplatin is available in two different dose vials containing 50 mg and 100 mg of active drug at a concentration of 5 mg/ml. It is stored at 25*C. It must be diluted prior to infusion in 250-500 ml of 5% dextrose [under no circumstance should 0.9% sodium chloride (NS) be used].

The drug will be prepared and infused, as is standard practice.

11.0 RESPONSE CRITERIA

Determination of response rate is a secondary objective of this study and any responses will be documented and reported. The RECIST criteria to determine treatment status (2000) will be followed.

11.1 Response Evaluation Criteria in Solid Tumors (RECIST)

Eligibility

Measurable disease - the presence of at least one measurable lesion. If the measurable disease is restricted to a solitary lesion, its neoplastic nature should be confirmed by cytology/histology.

Measurable lesions - lesions that can be accurately measured in at least one dimension with longest diameter >20 mm using conventional techniques or >10 mm with spiral CT scan.

Non-measurable lesions - all other lesions, including small lesions (longest diameter <20 mm with conventional techniques or <10 mm with spiral CT scan), i.e., bone lesions, leptomeningeal disease, ascites, pleural/pericardial effusion, inflammatory breast disease, lymphangitis cutis/pulmonis, cystic lesions, and also abdominal masses that are not confirmed and followed by imaging techniques; and.

All measurements should be taken and recorded in metric notation, using a ruler or calipers. All baseline evaluations should be performed as closely as possible to the beginning of treatment and never more than 4 weeks before the beginning of the treatment. The same method of assessment and the same technique should be used to characterize each identified and reported lesion at baseline and during follow-up.
Clinical lesions will only be considered measurable when they are superficial (e.g., skin nodules and palpable lymph nodes). For the case of skin lesions, documentation by color photography, including a ruler to estimate the size of the lesion is recommended.

Methods of Measurement -

CT and MRI are the best currently available and reproducible methods to measure target lesions selected for response assessment. Conventional CT and MRI should be performed with cuts of 10 mm or less in slice thickness contiguously. Spiral CT should be performed using a 5 mm contiguous reconstruction algorithm.

Baseline documentation of "Target" and "Non-Target" lesions

All measurable lesions up to a maximum of five lesions per organ and 10 lesions in total, representative of all involved organs should be identified as target lesions and recorded and measured at baseline. Target lesions should be selected on the basis of their size (lesions with the longest diameter) and their suitability for accurate repeated measurements (either by imaging techniques or clinically). A sum of the longest diameter (LD) for all target lesions will be calculated and reported as the baseline sum LD. The baseline sum LD will be used as reference by which to characterize the objective tumor. All other lesions (or sites of disease) should be identified as non-target lesions and should also be recorded at baseline. Measurements of these

lesions are not required, but the presence or absence of each should be noted throughout follow-up.

Response Criteria

Evaluation of target lesions
1. Complete Response (CR): Disappearance of all target lesions
2. Partial Response (PR): At least a 30% decrease in the sum of the LD of target lesions, taking as reference the baseline sum LD
3. Progressive Disease (PD): At least a 20% increase in the sum of the LD of target lesions, taking as reference the smallest sum LD recorded since the treatment started or the appearance of one or more new lesions
4. Stable Disease (SD): Neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD, taking as reference the smallest sum LD since the treatment started

Evaluation of non-target lesions
1.Complete Response (CR): Disappearance of all non-target lesions and normalization of tumor marker level
2.Incomplete Response/Stable Disease (SD): Persistence of one or more non-target lesion(s) or/and maintenance of tumor marker level above the normal limits
3.Progressive Disease (PD): Appearance of one or more new lesions and/or unequivocal progression of existing non-target lesion.

(1) Although a clear progression of "non target" lesions only is exceptional, in such circumstances, the opinion of the treating physician should prevail and the progression status should be confirmed later on by the review panel (or study chair).

Evaluation of best overall response

The best overall response is the best response recorded from the start of the treatment until disease progression/recurrence (taking as reference for PD the smallest measurements recorded since the treatment started). In general, the patient's best
response assignment will depend on the achievement of both measurement and confirmation criteria

Target lesions	Non-target lesions	Evaluation of NTL	Overall Response
CR	CR	No	CR
CR	Incomplete Resp./ SD	No	PR
PR	Non-PD	No	PR
SD	Non-PD	No	SD
PD	Any	Yes or No	PD
Any	PD	Yes or No	PD
Any	Any	Yes	PD

Patients with a global deterioration of health status requiring discontinuation of treatment without objective evidence of disease progression at that time should be classified as having "symptomatic deterioration". Every effort should be made to document the objective progression even after discontinuation of treatment.

In some circumstances it may be difficult to distinguish residual disease from normal tissue. When the evaluation of complete response depends on this determination, it is recommended that

the residual lesion be investigated (fine needle aspirate/biopsy) to confirm the complete response status.

Duration of overall response

The duration of overall response is measured from the time measurement criteria are met for CR or PR (whichever status is recorded first) until the first date that recurrence or PD is objectively documented, taking as reference for PD the measurements recorded since the treatment started.

Duration of stable disease

SD is measured from the start of the treatment until the criteria for disease progression are met, taking as reference the smallest measurements recorded since the treatment started.

11.2 Time to Disease Progression - From date of registration to date of progressive disease.

11.3 Duration of Response - Time from documentation of complete or partial response to documentation of disease progression.

11.4 Time to Death - From date of registration to date of death.

12.0 STATISTICAL CONSIDERATIONS

The main objective of the study is to determine extent of up regulation in the ERCC-1 gene expression on exposure to oxaliplatin *in vivo*. Specifically, in comparing PBMC with pre and post exposure to oxaliplatin (end of infusion – 2 hour sample), we hypothesize that ERCC-1 expression will be significantly increased after exposure. The baseline expression is relative to a standard protein, beta actin, and has been found to be 50%. Samples will be analyzed for ERCC-1 protein relative to beta-actin. A level of < 50% will be coded as negative (-), and that of > 50% will be coded as positive (+). A sample size of 40 pairs of observations (pre and post exposure) will give us 80% power to detect an odds ratio of 3.0 using a one-sided McNemar test with an alpha level of 0.025. This odds ratio is equivalent to a difference between paired proportions of 0.41 which assumes the proportion of samples with (+,-) pattern to be 0.20 and the proportion of (-,+)pattern to be 0.61. The proportion of discordant pairs is assumed to be 0.81. The study will accrue upto 80 patients to ensure enough yield for the 40 pairs of observations.

Comparison of before and after treatment ERCC-1 expression level will be compared using McNemar test. Response rate will be summarized as percentage with associated 95% exact confidence intervals. Time-to-event outcomes will be summarized using KM estimator as appropriate. Other continuous outcomes will be summarized using descriptive summaries such as mean, median, inter-quartile range, as appropriate.

13.0 CRITERIA FOR REMOVAL OF PATIENTS FROM PROTOCOL

When a patient is removed from study, the Principal investigator should be notified, and the reason for withdrawal noted in the flow sheets.

12.1 Progressive disease

12.2 Patient desire to withdraw

12.3 At the discretion of the Principal Investigator

14.0 DURATION OF STUDY

The study will last until the first tumor evaluation post treatment will be performed, at the end of 2 cycles [28 day cycle (2 doses of FOLFOX), or 21 day cycle, XELOX]. This will enable documentation of best response.

15.0 ADVERSE DRUG REACTION (ADR) REPORTING

Reports of adverse reactions will be made using the Modified NCI Common Toxicity Criteria for Adverse Events version 3.0 for reference according to the guidelines published by the NCI.

Report the following by telephone to the principal investigator or study coordinator at (718) 405-8404 or 718-405-8515, available 24 hours, [recorder after working hours]:

1. All unexpected grade 3 events
2. All life-threatening (Grade 4) events
3. All fatal events.

A full report to the PI is to be made within 10 days of the event.

The Institution Review Board of the Montefiore Medical Center will be notified of any SAE within 48 business hours of the time that the PI was informed of the event, by phone, fax or e-mail. A full report will be submitted within 10 business days of the time that the PI was informed of the event. If the event is ongoing, a follow up report will be made at the end of the event. All deaths within 6 months of discontinuation of study drugs will be reported. All SAE within 30 days of discontinuation of study drugs will be reported.

All serious adverse experiences/events (SAEs) that are unexpected and possibly related to use of the Study Drug (s) will also be reported directly to Sanofi-Aventis. The U.S. Package Insert shall be used to define expectedness.

See Appendix C for SAE form

16.0 REFERENCES

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16.0 LIST OF APPENDICES

Appendix A:

ECOG PERFORMANCE STATUS SCALE

0	Normal activity
1	Symptoms of disease, but ambulatory and able to carry out activities of daily living
2	Out of bed more than 50% of the time. Occasionally needs assistance
3	In bed more than 50% of the time, needs nursing care
4	Bedridden, may need hospitalization

Appendix B

ELIGIBILITY CHECKLIST

Patient Name: _____
Patient Medical Record #_____

All questions must be "yes" for patient to be eligible

1. Does the patient have a histological confirmed diagnosis of colon or rectal cancer? _____

2. Is the patient a candidate for oxaliplatin therapy _____

3. Is the patient > 18 years of age? _____

4. Is the Performance Status 0-2 ? _____

5. Does the patient have adequate organ function as defined? (all blood tests done within 14 days of registration)

	Parameter	Criteria	Date	Patient value
	WBC	> 3,000/μL		
	ANC	> 1500/μL		
	Platelet	> 75,000/μL		
	Creatinine clearance OR Creatinine	≥ 40 ml/mt ≤ 2 mg%		
	Bilirubin	≤ 2 mg/dl		
	SGOT/SGPT	≤ 3 X N		

6. Has the patient given informed consent? _____

7. Has she had a negative pregnancy test? _____

8. Is the patient at least 4 weeks out of major surgery, chemotherapy and 2 wk from radiation therapy and at least a week out of minor surgery? _____

Appendix C

sanofi-aventis U.S. Inc.
Global Pharmacovigilance and Epidemiology
200 Crossing Boulevard, P.O. Box 6890
Mailstop BX4-412-i
Bridgewater, NJ 08807

sanofi-aventis U.S. Inc.

Fax SAE REPORT

INVESTIGATOR SPONSORED TRIALS

Please √ one: ☐ **Eloxatin** ☐ **Taxotere** **Reported to FDA?** ☐ **Yes** ☐ **No**

Fax: **908-231-4827**

Date: **Pages:**

From: **Phone:**

IST#:	
Study Title:	
PI Name:	
Reportability:	All serious adverse/events (SAEs) unexpected and possibly related to the use of the Study Drug(s) are reported directly to the FDA in accordance with applicable law, regulations and Study protocol with a copy submitted to sanofi-aventis. If the FDA does not require the sponsor (investigator) to submit SAEs that are unexpected and related, SAE reports should be sent to sanofi-aventis when discovered. The U.S. Package Insert shall be used to define expectedness. All SAEs will be evaluated by the investigator for reportability. Relatedness is assessed using the definitions below. For Comparator Drugs / Secondary Suspects (Concomitant Medications), all serious adverse experiences will be forwarded to the product manufacturer.
Please √ one:	
	Unlikely: The event is clearly due to causes distinct from the use of the study drug, such as a documented pre-existing condition, the effect of a concomitant medication, a new condition which, based upon the pathophysiology of the condition, and the pharmacology of the study drug, would be unlikely related to the use of the study drug.
	Possible: The event follows a reasonable temporal sequence from administration of the study drug or the event follows a known response pattern to the study drug *BUT* the event could have been produced by an intercurrent medical condition which, based on the pathophysiology of the condition, and the pharmacology of the study drug, would be unlikely related to the use of the study drug or the event could be the effect of a concomitant medication.
	Probable: The event follows a reasonable temporal sequence from administration of the study drug and the event follows a known response pattern to the study drug *AND* the event cannot have been reasonably explained by an intercurrent medical condition *or* the event cannot be the effect of a concomitant medication.
	Definite: The event follows a reasonable temporal sequence from administration of the study drug, the event follows a known response pattern to the study drug and based on the known pharmacology of the study drug, the event is clearly related to the effect of the study drug.