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Sensitivity of Colorectal cancer cells to the recombinant Anthrax Lethal Toxin and the Urokinase- Activated Anthrax Lethal Toxin

By

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Dedication Page

"To my Beloved home SYRIA, I dedicate this work "

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Sensitivity of Colorectal cancer cells to the recombinant Anthrax Lethal Toxin

and the Urokinase- Activated Anthrax Lethal Toxin

ZEIN JEAN KASBO

ABSTRACT

In this study, we attempt to simultaneously target the major hallmarks of colorectal cancer (CRC), the MAPK pathway and the urokinase plasminogen activator system using a dual-selective, Urokinase-activated, recombinant anthrax lethal toxin (PrAgU2/LF). Colorectal cancer is the third most common cancer type in the world, and the second leading cause of death in the United States according to the American cancer society. Therefore, the need for alternative approaches is essential in order to overcome the difficulties faced with traditional therapies. PrAgU2/LF is composed of 2 components the binding moiety PrAgU2 and the catalytic moiety LF. PrAgU2 was obtained by replacing the furin cleavage sequence on PrAg with a urokinase- specific cleavage sequence. LF is a metalloprotease that cleaves and inactivates MEKs and hence inhibits the MAPK pathway. We assessed the cytotoxic effects and mechanisms of PrAgU2/LF on a panel of 9 human colorectal cancer cell lines.PrAg/LF, PrAg/FP59 and

PrAgU2/FP59 were used as controls for the different components of this system. PrAgU2/LF was not significantly cytotoxic to any of the cell lines tested, which were also resistant to PrAg/LF, indicating resistance to the inhibition of the MAPK pathway. This was further confirmed by resistance of these cells to the MEK1/2 inhibitor U0126. Notably, the majority of cell lines showed high levels of phospho-MEK1/2 but, surprisingly, no phospho-ERK1/2. Furthermore, targeting both the MAPK pathway and the PI3-kinase/Akt pathway also failed to induce cytotoxicity in colon cancer cell lines. In order to assess the possibility of targeting the uPA/uPAR system alone, we determined expression levels of uPAR and tested the sensitivity of CRC cells to PrAgU2/FP59, a urokinase activated, MAPK-independent version of the toxin. The vast majority of cell lines tested expressed uPAR and were sensitive to PrAgU2/FP59, indicating the possibility for targeting this protease system in CRC.

Keywords: CRC , Anthrax lethal toxin (PrAg/LF) , Urokinase system , MAPK, Dual-targeting.

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List of Abbreviations

Abbreviation	Full Name
CRC	Colorectal cancer
CMG-2	Capillary Morphogenesis Gene 2
CT scan	Computerized Tomography scan
ERK1/2	Extracellular Signal-Regulated Kinase 1 And 2
EF	Edema Factor
ET	Edema Toxin
FP59	Pseudomonas Aeruginosa Exotoxin A
FU	5-fluorouracil
GTP	Guanosine triphosphate
LF	Lethal Factor
LPS	Lipopolysaccharide
LV	Leucovorin
LT	Lethal toxin
LY294002	Potent inhibitor of PI3Ks
MAPK	Mitogen-Activated Protein Kinase
MAPKK	Mitogen-Activated Protein Kinase Kinase
MAPKKK	Mitogen-Activated Protein Kinase Kinasekinase
MEK1/2	Mitogen-Activated Protein Kinase Kinase
MRI	Magnetic Resonance Imaging
MMP	Metalloprotease
PAI-1	Plasminogen Activator Inhibitor- 1
PrAg	Protective Antigen

PrAg/LF	Recombinant anthrax lethal toxin
PrAgU2	Binding moiety of LF
PrAgU2/LF	Urokinase-activated, recombinant anthrax lethal toxin
PET scan	Positron emission tomography
PI3Ks	Phosphoinositide 3-kinases
RFI	Ratio of Fluorescence Intensity
TEM-8	Tumor Endothelium Marker 8
TNM classification system	Tumor / lymph Nodes/ Metastasis
uPA	Urokinase Plasminogen Activator
uPAR	Urokinase Plasminogen Activator Receptor
U0126	MEK1/2 inhibitor

CHAPTER ONE

INTRODUCTION

1.1.Colorectal cancer (CRC)

Colorectal cancer (CRC) is one of the most common types of cancer, being the third most prevalent type of tumor worldwide. Colorectal cancer, along with cancers of the lungs and prostate in men and of the lungs and breast in women, account for approximately 48% of cancer mortality worldwide. According to the American cancer society, colorectal cancer accounted for approximately 50,830 deaths in 2013 in the USA. A considerable decline in death rates due to colorectal cancer have been achieved recently due to advanced screening programs that lead to early detection and improvement in cancer diagnosis and therapy. Nevertheless not all demographic groups are able to benefit from these advances, partly because the decline in mortality is due to early detection and improvement of treatment, which are not readily available for all demographic groups (Siegel, 2013). A small proportion of colorectal cancer cases are genetically inherited while the vast majority of cases (90 to 95%) occur sporadically with the rates of CRC increasing with urbanization and industrialization. Etiology of the disease includes many factors such as smoking, prolonged use of non-steroidal anti-inflammatory drugs and genetic predisposition (Labianca, 2010).

In general, colorectal cancer starts in the form of small, benign adenomatous polyps, some of which become cancerous with time. In the early stages of the disease, small polyp-induced symptoms range from “no symptoms to very few non-specific symptoms” such as change in bowel habits, abdominal pain, unexplained weight loss and constant fatigue. Advanced symptoms include severe tiredness, anemia, obstruction in the bowel and metastases to liver resulting in hepatomegaly and jaundice in addition to a host of other symptoms. For these reasons, colorectal cancer is generally diagnosed either as a result of screening or if a patient presents with symptoms (Labianca, 2010).Diagnosis of CRC in usually carried out by endoscopy (colonoscopy) with a subsequent CT scan used to determine the extent of the tumor. Moreover, other imaging tests such as PET and MRI can be used. Once diagnosed, the next step is colorectal cancer staging, which is carried out in order to determine the degree of tumor penetration and the presence or absence of lymph nodes involvement using the TNM system. The TNM is a classification system that includes both the clinical and the pathological classification. However, the early diagnosis of peritoneal carcinomatosis using the various diagnostic tools currently available is still poor (Labianca, 2010).

Surgery remains the only curative therapeutic approach for localized colorectal cancer. However, metastatic cancer, which spreads to distant organs and tissues, is usually not curable and treatment is then directed to extend the patients' lifespan and improve his quality of life mainly via chemotherapy and palliative care. During surgical interventions, the perioperative milieu seems to facilitate the progression of metastatic colorectal tumors due to the exposure to lipopolysaccharide (LPS) contaminants that enter the systemic circulation due to bacterial gut translocation. It has been shown that

LPS enhance expression and activation of the urokinase plasminogen activator system that plays a crucial role in cancer metastasis (Killeen, 2009). Advanced stages of metastatic cancer usually involves combination or multiagentchemotherapy regimen (McLeod, 2010). Until recently, CRC first-line treatment plans include: 5-fluorouracil (FU) which is routinely administered along with leucovorin (FU/LV), or with irinotecan (IFL or FOLFIRI). The most common treatment plan includes the applicatoion of 5-fluoruracil (5FU)/LV and oxaliplatin (FOLFOX4). However, Increased susceptibility to infection due to severe neutropenia is one important side effect of this regimens that has been shown in 41.1% of patients. Moreover, chemotherapy has been shown to elevate oxidative stress levels which results in DNA, proteins, and cellular membrane damage in addition to spreading out the neoplastic cells (Santandreu, 2011).Therefore, the need for novel, potent and tumor-selective approaches for the treatment of colorectal cancer has become essential in order to overcome the difficulties faced in traditional therapies. Such novel approaches include targeting tumorigenic signaling pathways, such as the mitogen-activated protein kinase (MAPK) pathway, and cell surface proteases such as the urokinase plasminogen activator system (uPA/uPAR).

1.2. The MAP Kinase signaling pathway

Mitogen-activated protein kinases (MAPK) are a family of serine/threonine kinases that control important cellular processes such as proliferation, differentiation, growth, migration and cell death. This is carried out mainly by controlling gene expression, in particular through regulation of transcription. The MAPK pathway is

activated by extracellular growth signals such as growth hormones, epidermal growth factors and many mitogenic stimuli (Yang, 2013). To date, there are four major MAPK modules in mammalian cells which are the extracellular signal-regulated kinase 1 and 2 (ERK1/2) module, the extracellular signal-regulated kinase 5 (ERK 5) module, the stress-activated protein kinase 1 (JNK) module and the stress-activated protein kinase 2 (P38 pathway) module. Each MAPK pathway is comprised of a three-tiered cascade in which a mitogen-activated protein kinase kinasekinase (MAPKKK) phosphorylates and activates a mitogen-activated protein kinase kinase (MAPKK) which in turn phosphorylates and activates a mitogen-activated protein kinase (MAPK). Activation of the MAPKs results in their nuclear localization where they can phosphorylate and regulate transcription of a wide array of genes involved in vital biological processes. Upstream of the Kinase modules are members of the Rho and Ras families of GTPases that relay signals from receptor complexes to the modules. A recent genome-wide association study carried out by Lascorz et al. (2010) evaluated the genetic variations observed in the MAPK pathway in association with cancer and identified the MAPK-signaling pathway as one of the most important associated gene markers to several types of cancer including CRC. Hence, targeting the MAPK pathway is a tempting potential approach for the treatment of CRC. The association of mutations affecting the MAPK pathway and several types of tumors, including CRC is not surprising since we know that the different modules of the MAPK pathway are involved in fundamental cellular processes such as cell proliferation, differentiation and apoptosis, where abnormalities in MAPK signaling affect most if not all of these processes. Changes in the MAPK pathway may transform cells and enable them to acquire the critical capabilities of cancerous cells such as independence of proliferation signals, escape of apoptosis,

resistance to anti-mitotic signals, the capability to invade, metastasize and sustain angiogenesis. (Dhillon, 2007) The most frequent mitogenic mutations of MAPK pathway affect the Ras–Raf axis in the ERK pathway suggesting it functions as a regulatory hotspot of the MAPK pathway.

In this context, the ERK pathway has attracted attention for drug discovery for the past 15 years by targeting Ras, Raf and MEK1/2 mainly. Hence, a main focus of research today is the development of MAPK inhibitors and the assessment of their efficacy and selectivity in cancer therapy. Moreover, it has been recently shown that targeting downstream effectors can result in increased specificity and decreased side effects by eliminating only the targeted functions, thus ERK inhibitors are currently being actively developed. Hence, novel inhibitors of the Ras-Raf-MEK1/2-ERK1/2 branch of the MAPK pathway are urgently needed. A recently identified, and actively investigated, potent inhibitor of the MAPK pathway is anthrax lethal toxin (PrAg/LF).

1.3. Anthrax Lethal Toxin

Anthrax lethal toxin is produced by the gram-positive, spore-forming bacterium *Bacillus anthracis*, the causative agent of anthrax infections. Two major virulence factors are secreted by *B. anthracis*: the anthrax lethal toxin and the anthrax edema toxin. Anthrax toxin proteins are encoded on an extra chromosomal plasmid pXO1 (182 kb). The toxin components are: the protective antigen (PrAg, 83 kDa), the lethal factor (LF, 89 kDa) and the edema factor (EF, 90 kDa). Each factor alone is non-toxic, however they combine to form the different anthrax toxins, where lethal toxin (LT) is

composed of a combination of PrAg and LF, whereas edema toxin (ET) is composed of a combination of PrAg and EF. Protective antigen (PrAg) functions as the binding and internalization moiety while lethal factor (LF) and edema factor (EF) function as the catalytic moieties. Lethal factor (LF) is a zinc-dependent metalloprotease that cleaves and inhibit the mitogen-activated protein kinase kinases (MEKs) 1, 2, 3, 4, 6 and 7 resulting in the inactivation of the 3 major MAPK pathways: the extracellular-signal-regulated kinase via MEK 1 and 2, the p38 pathway via MEK 3 and 6, and the Jun N-terminus kinase (JNK) through MEK 4 and 7. Edema factor is a calmodulin-dependent adenylyl cyclase that leads to an increase in the intracellular levels of cAMP leading to variable responses such as tissue damage and death. Two anthrax toxins receptors were identified: The tumor endothelial marker 8 (TEM8) and the capillary morphogenesis gene-2 (CMG2) also referred to as anthrax toxin receptors 1 and 2, respectively. The physiological functions of the 2 receptors are not yet fully understood. However, studies have shown TEM8 up regulation in human colorectal cancer suggesting it to be a potential target for cancer therapy. Moreover, CMG2 was also suggested to have a role in angiogenesis (Liu, 2014).

1.4. Anthrax lethal toxin mechanism of action

Protective antigen (PrAg) binds to its membrane receptor on target cells (TEM8 and CMG-2) and is proteolytically cleaved by ubiquitously-expressed furin-like proteases at a specific sequence 164RKKR167 generating the 63 kDa receptor-bound fragment $PrAg_{63}$ and the 20 kDa free fragment $PrAg_{20}$. Release of $PrAg_{20}$ activates

$PrAg_{63}$ which oligomeraizes (both the heptamer and octamer forms have been observed) allowing the binding of three to four molecules of LF. The $PrAg_{63}$ oligomer/LF complex then undergoes clathrin-dependent receptor-mediated endocytosis (Liu, 2014).

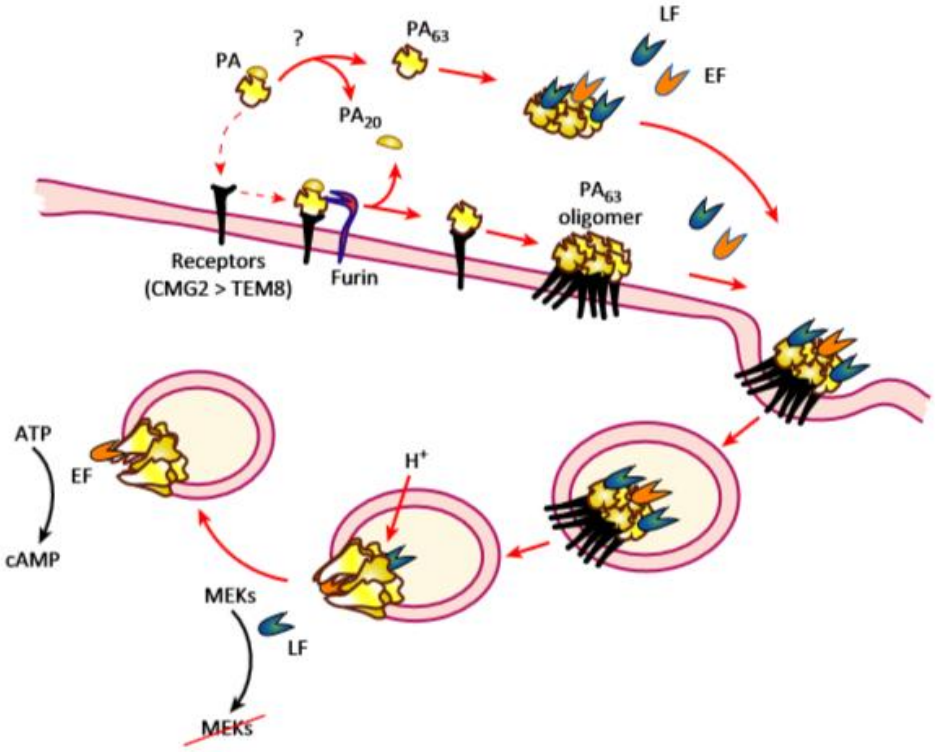


Figure 1.1 : Anthrax lethal toxin mode of action (Shihui Liu, MahtabMoayeri, and Stephen H. Leppla, 2014). Protective antigen (PrAg) binds to its membrane receptor on target cells (TEM8 and CMG-2) and is proteolytically cleaved by furin-like proteases at a specific sequence $_{164}$RKKR$_{167}$ generating the $PrAg_{63}$ activereceptor-bound fragment and the $PrAg_{20}$ free fragment. $PrAg_{63}$heptamerizes allowing the binding of three to four molecules of LF. The $PrAg_{63}$ oligomer/LF complex then undergoes endocytosis. Upon acidification of late endosomes LF translocate into the cytosol. LF is a Zinc-metalloprotease that cleaves and inactivates MEKs leading to the inhibition of MAPK pathways.

The translocation of LF from the endosome to the cytosol occurs following one of two mechanisms. In the first mechanism, considered by far as the most common, upon acidification of the late endosome, the $PrAg_{63}$ pre-pore oligomer undergoes a conformational change leading to the formation of a channel through which LF escapes the endosome and translocates into the cytosol of the cell. In the second mechanism, LF may be translocated into the intraluminal vesicles in order to be transported to the endosome through multivesicular bodies. Once inside the endosome, back fusion of the intraluminal vesicles with the limiting membrane delivers LF to the cytosol. LF is a Zinc-metalloprotease that cleaves and inactivates MEKs leading to the inhibition of MAPK pathways (Liu, 2014). In this context, MAPK-dependent tumors could be selectively vulnerable to anthrax lethal toxins. Several studies have recently shown the potency and tumor-selectivity of recombinant anthrax lethal toxin in a number of MAPK-dependent tumor types both on cell lines and in animal models. An in vitro study by Koo et al. (2002) and by Abi-Habib et al. (2005) demonstrated potency, selectivity and mechanism of action of anthrax lethal toxin on melanoma cells (Abi-Habib. 2005). Moreover, an in vivo study was done by the same group in 2006 demonstrating the *in vivo* potency of the toxin and showing that systemic treatment of a human melanoma xenograft model with PrAg/LF yielded complete tumor regression with minor cytotoxicity to mice, justifying further preclinical studies (Abi-Habib, 2006). Another study by huang et al. (2008) showed Fusion anthrax lethal toxin also exhibit a potential capability of inhibiting the MAPK pathways in renal cell carcinoma that have increased expression levels of both MKK1 and ERK2 and resulting in cell death. In 2013, Kassab et al. demonstrated PrAg/LF has a significant cytotoxic effect on MAPK-dependent AML cells (Kassab, 2013).

1.5. The Urokinase Plasminogen Activator protease System (uPA System)

Expression of the urokinase plasminogen activator protease (uPA/uPAR) system is one important hallmark of invasive and metastatic tumors. It is highly expressed in many types of solid and hematological malignancies and at the same time is rarely expressed on normal cells (Abi-Habib, 2006). Invasiveness and metastasis is considered a multi-step process that alters adhesion and physical movement of the cell. This requires the degradation of the basement membrane (BM) and the extracellular matrix (ECM), which carried out by specific cell surface proteases including Matrix metalloproteases (MMPs) and the urokinase plasminogen activator protease system (uPA/uPAR) (Lu, 2012). In colorectal cancer, excessive degradation of the ECM is attained via the matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9), and urokinase-type plasminogen activator (uPA/uPAR) (Kim, 2006). Thus, the uPA/uPAR system expression is considered as a marker for malignancy and a bad prognostic marker in tumors including CRC. The urokinase plasminogen activator system is involved in the control of matrix integrity and cell signaling resulting in different responses, under physiological and/or pathological conditions.

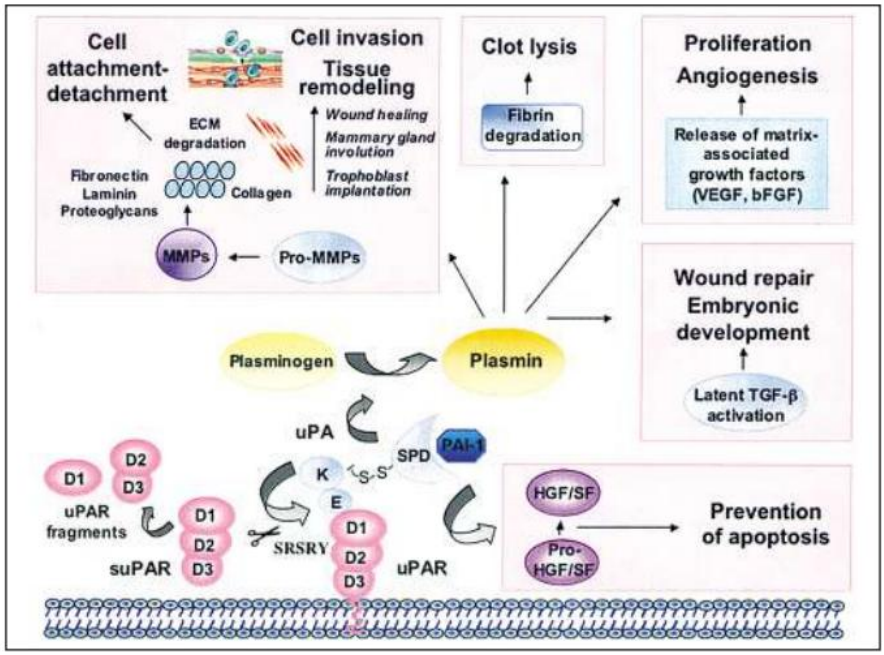


Figure 1.2. Different effects of uPA system on cell signaling, proliferation and Extracellular matrix degradation (Daniela Alfano et al., 2005). The uPA system consists of the urokinase-type plasminogen activator (uPA), specific membrane receptor (uPAR), and the plasminogen activator inhibitors (PAIs). The inactive enzyme binds to the receptor and becomes activated upon cleavage by plasmin and conversion into the active uPA. Active uPA then converts the zymogen plasminogen to the active plasmin. plasmin in turn leads to the activation of matrix metalloproteases, leading to the massive degradation and loss of integrity of the extracellular matrix. And regulation of fibrin degradation hence clot lysis. Also release of VEGF, bFGF and TGF-β factors which have role in proliferation and wound healing processes.

The uPA system consists of the urokinase-type plasminogen activator (uPA) and its own specific membrane receptor (uPAR), in addition to the plasminogen activator inhibitors (PAIs) (Tang, 2013). The urokinase plasminogen activator is secreted in the form of inactive enzyme (pro-uPA) and becomes activated following cleavage by plasmin and conversion into the active enzyme uPA. Active uPA then converts the

zymogen plasminogen to the active serine protease plasmin, starting a positive feedback loop and providing the cell with surface protease activity capable of proteolytic degradation of the extracellular matrix and of regulation of matrix integrity via the activation of pro-metalloproteinases (pro-MMPs) into active metalloproteases (MMPs) (Alfano, 2005). Hence, by leading to the activation of plasmin, the uPA/uPAR system starts a positive feedback loop allowing the generation of active plasmin, which, in turn, leads to the activation of matrix metalloproteases, leading to the massive degradation and loss of integrity of the extracellular matrix, a pre-requisite for cancer invasion and metastasis. uPA receptor (uPA-R) is a 50-65-kD glycosylphosphatidylinositol (GPI)-anchored protein. uPAR binds to both inactive pro-uPA and active uPA, binding of uPA to uPAR stabilizes the molecule and protects it from inhibition by plasminogen activator inhibitors (PAIs), consequently leading to more plasminogen being converted to active plasmin. In the absence of uPAR, active uPA is rapidly inhibited by PAIs, hence the need for the presence of both components uPA and uPAR to achieve an active urokinase plasminogen activator system. The glycoproteins plasminogen activator inhibitors (PAI 1 and PAI 2) regulate the uPA system. They inhibit both free and receptor-bound uPA, with the inhibition constant for receptor bound uPAR being several hundred folds higher (Kim, 2006). Under normal conditions, plasma and tissue concentrations of PAIs are very low. However, their levels increase under pathological conditions. PAI-1 plays an important role in the recycling of the urokinase system components. PAI-1 binds to uPA/uPAR resulting in the formation of a (uPA-R/uPA/ PAI-1) complex. The complex then undergoes internalization and breaks down into 2 fractions, the uPA/PAI-1 and uPA-R. The uPA/PAI-1 complex is degraded in the lysosome while the uPA receptor is recycled to the cell surface.

Studies have shown that increased levels of PAI-1 are associated with metastatic cancers in comparison with non-metastatic cancers. Thus, PAI 1 along with uPA, are considered poor prognostic markers (Kim, 2006). The uPA system, as noted previously, is not normally expressed under physiological conditions, except during particular processes such as wound healing or tissue remodeling (Andreasen, 2000). A study by Tae-Dong Kim in 2006 showed that colorectal cancer tissues have significantly higher levels of uPA and PAI-1 when compared to corresponding normal mucosal tissues. High levels of uPA and uPAR were observed in 100% (22/22) of patient colorectal tissues (Kim, 2006). Considering all these facts, the uPA system was and still is of great interest as a selective, potential marker of cancer cells with minimal expression on normal healthy cells. One study in 2004 by Abi-Habib et al., attempted to take advantage of the overexpression of uPA/uPAR on AML cells to generate a urokinase-activated, dual-specific fusion toxin, DTU2GMCSF. This toxin consists of the catalytic and translocation domains of diphtheria toxin (DT) and the granulocyte macrophage colony-stimulating factor (GMC-SF). The furin cleavage site on diphtheria toxin was replaced by a uPA cleavage site termed U2 in order to enhance specificity to uPA/uPAR expressing AML cells. The single targeting moiety using DT_{388}GMCSF showed clinical efficacy but also showed cross-reactivity with GMCSF-expressing normal cells. However, DTU2GMCSF, the modified, urokinase-activated fusion toxin with dual specificity exhibited a significantly increased specificity and reduced normal cells toxicity while retaining potency and range (Abi-Habib, 2004).

1.6. The dual- selective recombinant toxin PrAgU2/LF

In PrAgU2, the furin cleavage site of PrAg ($_{164}$RKKR$_{167}$) has been replaced by a urokinase-specific cleavage site termed U2 ($_{163}$PGSGRSA$_{169}$). By this modification, the new engineered fusion toxin theoretically should bind to all cells through the anthrax toxin receptors; however, it will get activated only on tumor-cells expressing an active uPA/uPAR system. Upon binding and activation by urokinase, the toxin complex would undergo endocytosis and eventually release LF into the cytosol. Once in the cytosol, LF inactivates MEKs and hence the MAPK pathways leading to proliferation inhibition and rapid cell death in MAPK-dependent cancer cells.

1.7.Targeted fusion protein toxins:

Recombinant anthrax lethal toxin (PrAg/LF) and its urokinase-activated version (PrAgU2/LF) belong to the category of targeted cancer therapeutics, and more specifically to the category of fusion toxins. Fusion Toxins are recombinant proteins that are genetically modified to specifically target tumor cells. These targeted toxins are composed of a targeting polypeptide ligand associated with a bacterial or plant toxin. Fusion toxins represent a novel class of tumor-specific therapeutics that aim to both enhance malignant cell specificity and minimize undesirable cytotoxicity to normal cells. A number of these genetically modified fusion toxins have been developed under good manufacturing practice and reached clinical trials. Despite the multiple hurdles of working with such a complex molecules, promising results were obtained to date

(Frankel.2000). The fusion toxin may target a tumor specific cell receptor or marker and the toxin moiety would enter the cell and induce cell death such in the case of DTGMCSF (Diphtheria toxin granulocyte-macrophage colony-stimulating factor) and DT_{388}IL3 (Diphtheria toxin interleukin 3), among others. These two recombinant toxins target the GMCSF receptors and IL3 receptors, respectively on leukemic cells (Frankel, 2008) (Abi-Habib, 2004) while the toxin moiety (DT) results in cell death by inhibiting protein synthesis. On the other hand, the fusion toxin may target specific pathways that are up-regulated in tumor tissues via the toxin moiety such as Anthrax lethal toxin (PrAg/LF). In this case, PrAg, the binding and internalization moiety binds to the anthrax lethal toxin receptors 1 & 2 and mediates internalization of lethal factor LF which will inhibit the MAPK pathway, a major hallmark of tumor cells. This inhibition will results in cell cycle arrest and cell death (depending on the tumor type) leading to tumor regression (Kassab, 2013).

Recently, a new category of recombinant toxins with dual-targeting capabilities have been developed such as DTU2GMCSF, where, in addition to targeting the GMCSF receptors it targets the urokinase plasminogen activator (uPA) system which is a tumor-specific protease. Therefore, enhanced tumor specificity and less normal tissue damage can be obtained (Abi-Habib, 2004).PrAgU2/LF is another dual selective-tumor fusion protein toxin. It targets MAPK dependent tumor cells that are at the same time express the tumor-specific urokinase plasminogen activator proteases. PrAgU2/LF induces the inhibition of MAPK pathways and results in inhibition of cell proliferation and apoptosis.Hence, in this study, we attempt to target the two major hallmarks of colorectal cancer, the MAPK pathway and the urokinase plasminogen activator system

by modifying the furin-activation site of PrAg into a urokinase-activation site termed U2. Hence, the resulting toxin PrAgU2/LF is a dual-specificity, urokinase-activated recombinant anthrax lethal toxin, which would require expression of uPA/uPAR and dependence on the MAPK pathway for activity.

CHAPTER TWO

MATERIALS AND METHODS

2.1. Expression and purification of PrAg, LF and FP59

Recombinant anthrax proteins, PrAg (activated by furin), PrAgU2 (activated by the urokinase plasminogen activator), LF and FP59 (fusion of the PrAg binding domain of LF and the catalytic domain of Pseudomonas aeruginosa exotoxin A, which inhibits protein synthesis and induces MAPK-independent cell death), were expressed and purified in the laboratory of Stephen H. Leppla at the National Institute of Allergies and Infectious Diseases (NIAID) of the National Institutes of Health (NIH) in Bethesda, MD, as described previously (Ramirez DM, 2002) (Liu S, 2001). Briefly, fermentation was carried out by inoculating a 12 to 14 h old starter culture grown from a frozen stock. Three to ten liter fermentations were carried out controlling dissolved oxygen (DO) at 30% saturation, temperature at 37^{o}C, and pH at 7.5. At harvest time, 5 mM EDTA and 10 ug/ml PMSF (phenylmethylsulfonyl fluoride) were added to the culture. Purification was then carried out as follows: 1. Packed-bed hydrophobic interaction chromatography during which the cell suspension was centrifuged and the supernatant was passed through a 0.2 -µm hollow fiber filter (AGT, Needham, MA). The filtered broth was then concentrated 20 fold using a 10K membrane. After sample loading, the column was washed with 10 column volumes (CV) of equilibration buffer and rPA was eluted with a 30 CV linear gradient of (NH4)2SO4 in 10 mM HEPES, 5 mM EDTA. The rPA-

containing fractions were pooled for further purification. 2-Expanded-bed hydrophobic interaction chromatography in which the cell suspension was diluted 1:1 with buffer and the diluted cell suspension was loaded upward at 300 cm/ h. The column was washed in expanded mode with 10 CV of equilibration buffer. Elution was performed in packed-bed mode with 8 CV of elution buffer at 100 cm/ h. 3- Anion exchange chromatography in which fractions from HIC were dialyzed against 20 mMTris pH= 8.9/ 5 mM EDTA and loaded on a Q Sepharose Fast Flow (Amersham Pharmacia Biotech) column. The protein was eluted using a 20 CV linear gradient of NaCl in the same buffer. rPA-containing fractions were concentrated and dialyzed against PBS. (Ramirez DM, 2002) (Liu S, 2001). LY294002 (PI3K inhibitor) was purchased from Cell signaling Technology (Danvers, MA). U0126 (MEK1/2 inhibitor) was purchased from Cell signaling Technology (Danvers, MA).

2.2. Cells and cell lines

Nine human Colorectal cancer cell lines SW1116, SW837, SW948, LoVo, Caco-2, HT-92, T-84, Sk-co1 and SNU-c1 were obtained from the American Type Culture Collection (ATCC) and grown in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin. Cells were maintained in a water-jacketed CO2 incubator at 37^{o} C, 5% CO2.

2.3. Proliferation inhibition assay (Cytotoxicity)

Sensitivity of CRC cell lines to PrAg/LF and PrAgU2/LF was determined using a proliferation inhibition assay as described previously (Abi-Habib RJ, 2005) We have also used a recombinant protein, termed FP59, consisting of the PrAg binding domain of LF fused to the catalytic domain of *Pseudomonas aeruginosa* exotoxin A. Binding of FP59 to PrAg or PrAgU2 and its translocation into the cytosol are identical to those of LF, however, it does not target the MAPK pathway but rather ADP-ribosylates elongation factor 2 (EF-2) leading to inhibition of protein synthesis and cell death. Hence, FP59 is a MAPK-independent targeting moiety. PrAg/FP59 was used as a control for catalytic domain entry into the cytosol of CRC cells, whereas PrAgU2/FP59 was used as a control for the cell surface activity of the urokinase plasminogen activator independently of cell sensitivity to the inhibition of MAPK pathways Briefly, aliquots of 10^4 cells/well, in 100 µl cell culture medium, containing a fixed concentration of 10^{-9} M LF or FP59, were plated in a flat-bottom 96-well plate (Corning Inc. Corning, NY). Then, 50 µl PrAg or PrAgU2 in media were added to each well to yield concentrations ranging from 10^{-8} to 10^{-13} M. Following a 48 h incubation at 37oC/5% CO2, 50 µl of XTT cell proliferation reagent (Roche, Basel, Switzerland) were added to each well and the plates incubated for another 4 h. Absorbance was then read at 450 nm using a microplate reader (Thermo Fisher Scientific, Waltham, MA). Nominal absorbance and percent maximal absorbance were plotted against the log of concentration and a non-linear regression with a variable slope sigmoidal dose-response curve was generated along with IC_{50} using GraphPad Prism 5 software (GraphPad Software, San Diego, CA).

All assays were performed at least twice with an inter-assay range of 30% or less for IC_{50}.

2.4. Cell cycle analysis

The impact of treatment on the cell cycle of CRC cells was determined using Propidium Iodide (PI)-staining on flow cytometry. Briefly, cells incubated with PrAg/LF (10,000 pM), PrAg/FP59 and LF alone as a control in a 6-well plates (Corning Inc. Corning, NY) for 48 and 72 h at 37oC, 5% CO2, were harvested and fixed in 70% ethanol for a minimum of 24 h, at -20^{o}C. Cells were then incubated in 500 µl PI staining solution (50 µg/ml) for 40 min at 37^{o}C. Samples were then read on a C6 flow cytometer (BD Accuri, Ann Arbor, MI) and total cell DNA content was measured on FL2-A. Percent of cells in G0/G1, S and G2/M phase was determined in control cells and in cells treated with PrAg/LF and with PrAg/FP59 following gating for the cell population on width versus forward scatter.

2.5. Inhibition Assays

CRC cells were incubated with either the small molecular weight PI3 kinase (PI3K) inhibitor LY294002 (Cell Signaling Technology, Danvers, MA) alone and in combination with PrAg/LF or with the small molecular weight MEK1/2 inhibitor U0126 (Cell Signaling Technology, Danvers, MA). Briefly, 10^4 cells/well were plated in 100 µl

of medium in a flat-bottom, 96-well plate. Then 100 µl of either medium (control cells) or medium containing LeTx (10^{-8}M PrAg/10^{-9}M LF), LY294002, U0126 or a combination of the above were added. When LY294002 or U0126 were used, they were added as described above for PrAg or PrAgU2 but in concentrations ranging from 10^{-4} to 10^{-9} M. Cells were then incubated for 48 h at 37oC, 5% CO2 followed by the addition of 50 µl of XTT cell proliferation reagent (Roche, Basel, Switzerland). Cells were incubated for another 4 h and absorbance was read at 450 nm using a 96-well plate reader (Thermo Fisher Scientific, Waltham, MA). Data was analyzed using GraphPad Prism V software (GraphPad Software, San Diego, CA). The absorbance and the percent absorbance of controls were compared between the different treatment groups.

2.6. Intracellular Staining and Flow Cytometry Analysis

Active MEK1/2, ERK1/2 and AKT in untreated CRC cell lines was assessed by determining the presence or absence of phosphorylated form of each of MEK1/2, ERK1/2 and AKT using flow cytometry as described previously. Approximately $3x10^6$ cells were fixed in 70% ethanol for 15 min. cells were resuspended in antibody binding buffer containing 0.05% Triton-X 100, and incubated with a 5µl of anti-phospho-ERK1/2 (Ser 217/221) rabbit monoclonal antibodies (Cell Signaling Technology, Danvers, MA) or anti-phospho-MEK1/2 or anti-phospho-AKT, for 1 h at 37^{o}C, followed by a 30-minute incubation with a 1/100 dilution of a FITC-conjugated mouse anti-rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Fixed cells stained only with FITC-conjugated mouse anti-rabbit polyclonal antibody were used as

isotypic control. Samples were then analyzed using a C6 flow cytometer (BD Accuri, Ann Arbor, MI). The presence of p-ERK1/2, p-MEK1/2, p-AKT was analyzed and compared with that of the isotopic control. Positivity for the presence of p-ERK1/2, p-MEK1/2 or p-AKT was determined using the ratio of fluorescence intensity (RFI) between the mean fluorescence intensity (MFI) of the stained cells and the MFI of the isotypic control. RFI > 2 was considered positive, RFI between 1.5 and 2 were considered slightly positive and RFI <1.5 was considered negative.

2.7. Expression of uPAR

Expression of the urokinase plasminogen activator receptor (uPAR) on CRC cell lines was determined using single cell staining with a FITC-conjugated anti-uPAR antibody on flow cytometry as described previously. Approximately 10^6 cells were incubated with a 1/100 dilution of a FITC-conjugated anti-uPAR mouse monoclonal antibody (Cell Signaling Technology, Danvers, MA) in antibody binding buffer for 1 h at 37oC. Cells stained only with a FITC-conjugated mouse IgG were used as isotypic control. Samples were then analyzed using a C6 flow cytometer (BD Accuri, Ann Arbor, MI). The expression of uPAR was analyzed on FL1-H and compared with that of the isotopic control. Positivity for the presence of uPAR was determined using the ratio of fluorescence intensity (RFI) between the mean fluorescence intensity (MFI) of the uPAR stained cells and the MFI of the isotypic control. RFI > 2 was considered positive. RFI between 1.5 and 2 were considered slightly positive and RFI <1.5 was considered negative.

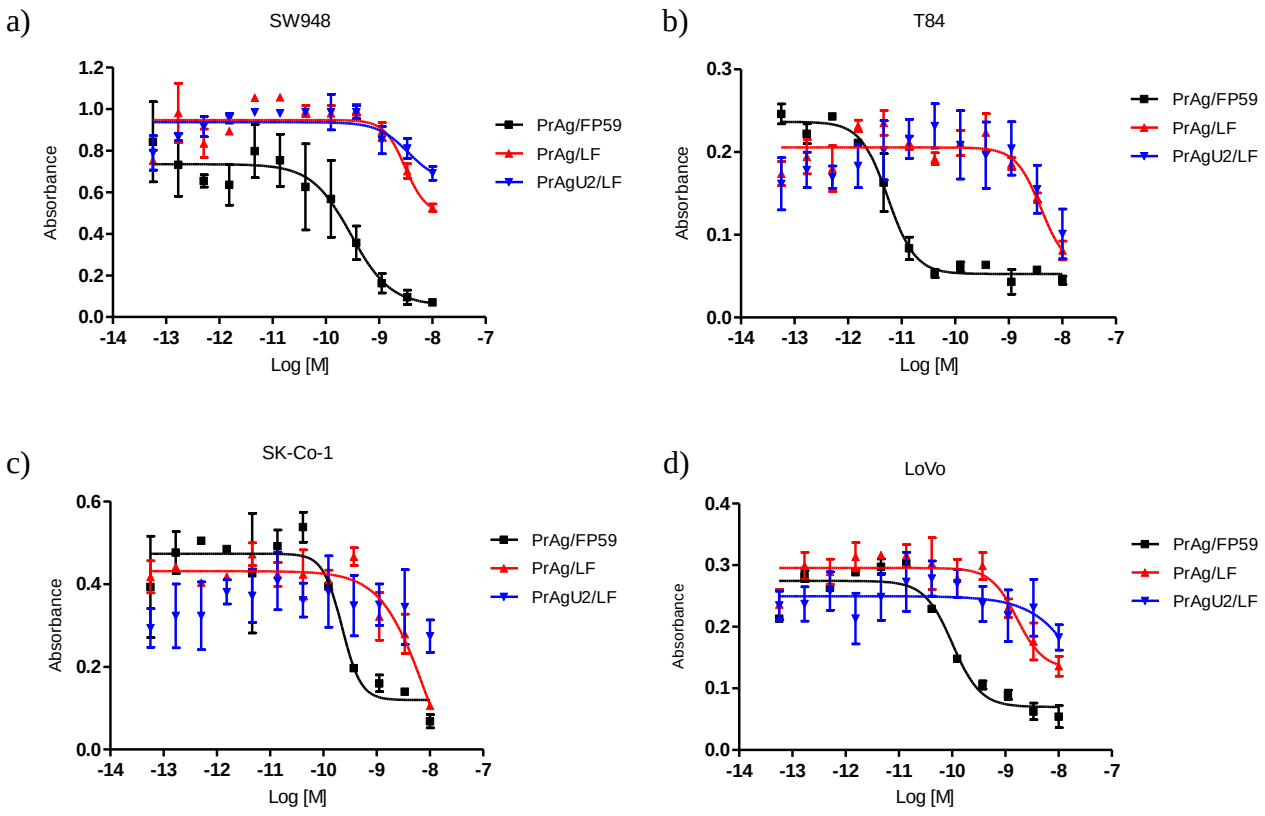
CHAPTER THREE

RESULTS

3.1. CRC cell sensitivity to PrAg/LF and PrAgU2/LF

A panel of nine human CRC cell lines was tested for sensitivity to the recombinant anthrax lethal toxin PrAg/LF and its urokinase-activated version PrAgU2/LF. Cell viability following treatment was assessed using XTT proliferation inhibition assay which detects metabolically active cells after incubation with the toxin. PrAg/LF is activated by furin-like proteases and induces cell death via the inhibition of the MAPK pathway, whereas PrAgU2/LF is activated by the urokinase plasminogen activator system and also target the MAPK pathway. Only 1 of 9 cell lines tested (SNU-c1) was sensitive to PrAg/LF with none being sensitive to PrAgU2/LF (IC50 >10,000 pM) (Figure 3.1).To understand whether the resistance to PrAg/LF and PrAgU2/LF was due to cell resistance to the LF-mediated inactivation of MAPK pathway or due to the absence of anthrax toxin receptors, we tested potency of PrAg/FP59 as a control. FP59 is a fusion protein consisting of the PrAg binding domain of LF and the catalytic domain of *Pseudomonas aeruginosa* exotoxin A. Binding of FP59 to PrAg leads to FP59 translocation into the cytosol just like LF, however, FP59 does not target the MAPK pathway but instead ADP-ribosylates elongation factor 2 leading to the inhibition of protein synthesis and cell death. FP59, therefore, is toxic to all cells once translocated

into the cytosol. Thus, PrAg/FP59 serves as a control for the presence or absence of anthrax toxin receptors and, in more general terms, the ability of the catalytic moiety to access the cytosol. As illustrated in Table 3.1 and Figure 3.1, 4 out of the 9 cell lines tested were not sensitive to PrAg/FP59 (HT-29, SW837, CaCo-2 and SW116), indicating that resistance to PrA/LF and, subsequently, to PrAgU2/LF of these cell lines is due to absence of anthrax toxin receptors and inability to internalize LF. The remaining 5 cell lines, were sensitive to PrAg/FP59, hence the resistance of 4 of them to PrAg/LF and PrAgU2/LF (T84, LoVo, Sk-Co-1 and SW948) was due to their resistance to the LF-mediated inhibition of the MAPK pathway (Table 3.1).



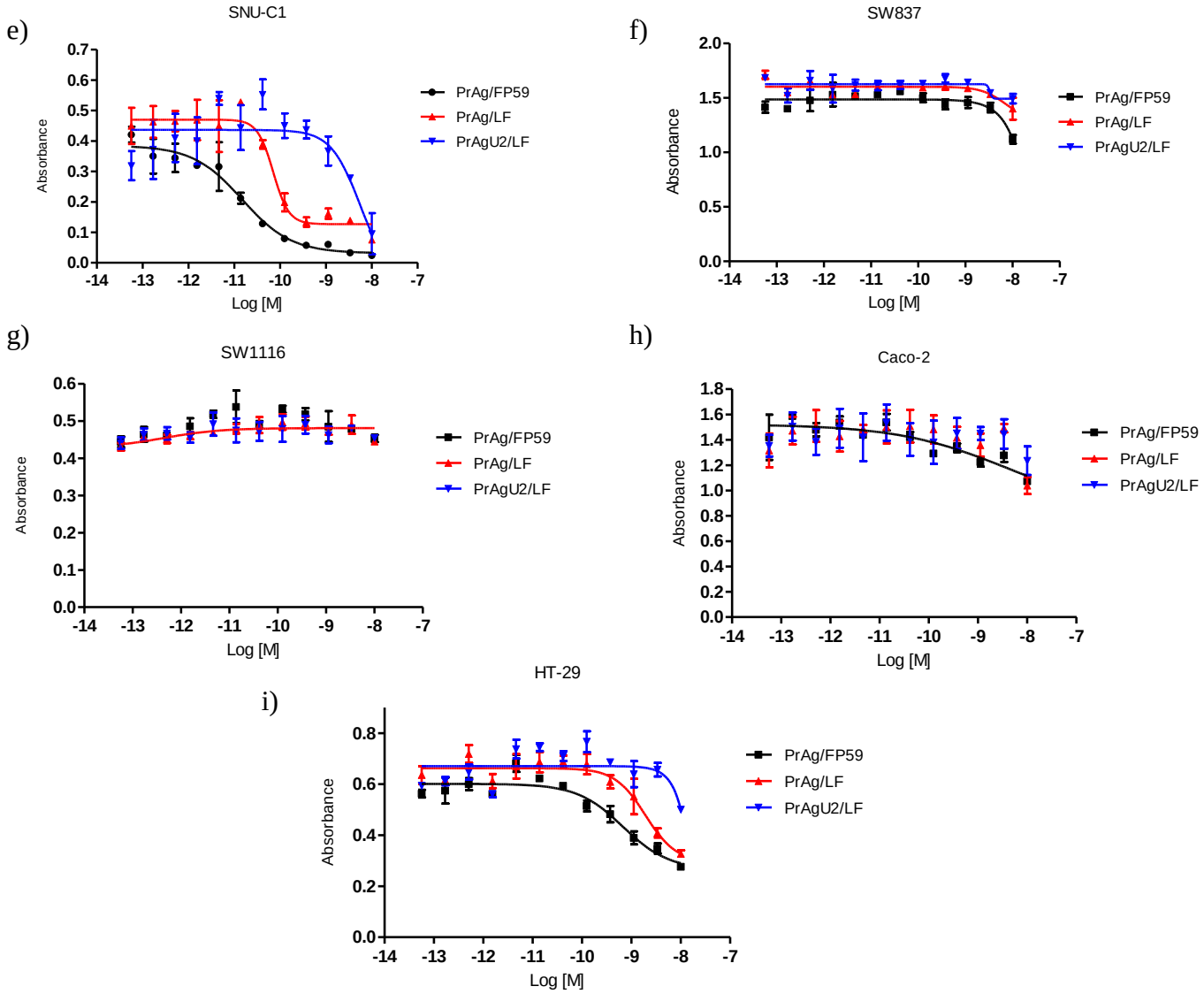


Figure 3.1. Sensitivity of human CRC cell lines to PrAg/LF, PrAgU2/LF and PrAg/FP59determined by a proliferation inhibition assay. Only one cell line, SNU-c1(e) is sensitive to the treatment with PrAg/LF, whereas all the other 8 cell lines were not sensitive (a,b,c,d,f, g and i). None of the cell lines is sensitive to PrAgU2/LF (a, b, c, d, e, f, g and i). SW948(a), T-84(b), LoVo (d) and SNU-c1(e) are sensitive to PrAg/FP59. X-axis, log of the molar drug concentration, Y-axis, cell viability.

Table 3.1. Sensitivity of human CRC cell lines to PrAg/LF, PrAgU2/LF and PrAg/FP59.

Cell line	PrAg/LF (IC_{50}, pmol/L)	PrAgU2/LF (IC_{50}, pmol/L)	PrAg/FP59 (IC_{50}, pmol/L)
SNU-c1	71	8060	15
T84	3950	>10000	6.0
LoVo	1641	>10000	101
Sk-Co-1	8120	>10000	222
SW948	3041	3478	N/A
HT-29	>10000	>10000	>10000
SW837	>10000	>10000	>10000
Caco-2	>10000	>10000	>1000
SW1116	>10000	>10000	>10000

N/A: Not available

Since cells that are resistant to PrAg/FP59 have no anthrax toxin receptors and thus, were unable to translocate LF into the cytosol, we needed an alternative method to test for their sensitivity to the inhibition of the MAPK pathway. For this purpose, we tested sensitivity of cells to the specific MEK1/2 small molecular weight inhibitor U0126. None of the CRC cell lines tested was sensitive to the U0126-mediated inhibition of the MAPK pathway further confirming the resistance of this tumor type to the targeted inhibition of MEKs (Figure 3.2).

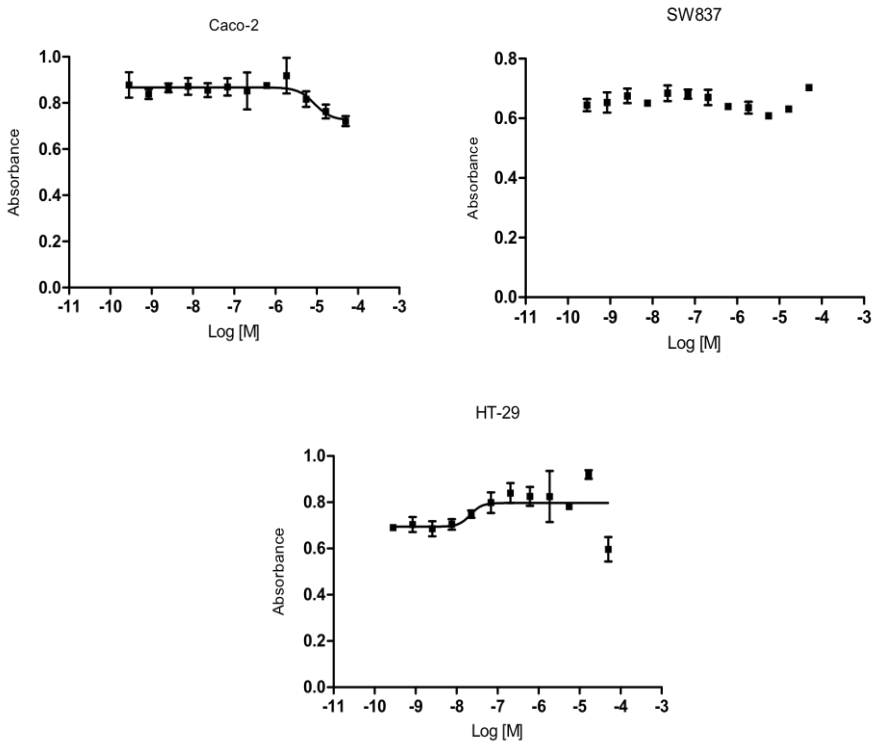
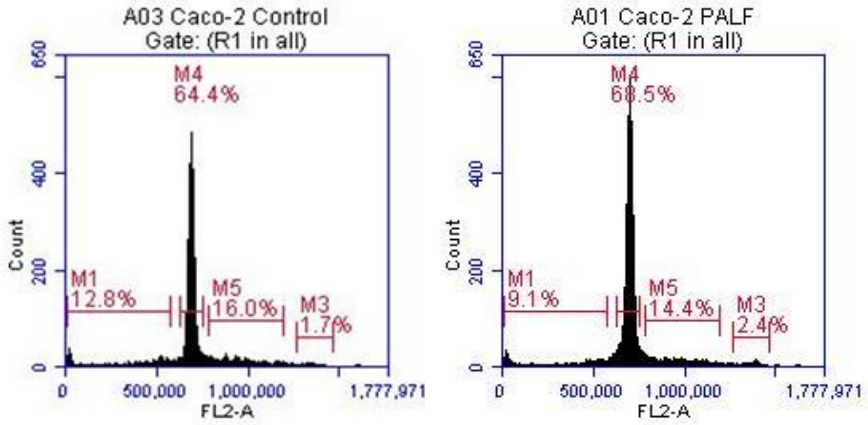


Figure 3. 2. Effect of MAPK pathway inhibition using the MEK 1/2 inhibitor U0126 on CRC cell lines. None of the tested CRC cell lines showed sensitivity to the U0126-mediated inhibition.

3.2. Cell cycle effect of PrAg/LF

After assessing cytotoxicity of MAPK inhibition to CRC cell lines, we wanted to assess the effects of the inhibition of this pathway on the cell cycle of CRC cells and whether PrAg/LF has any cytostatic effect in this tumor type. Cell cycle analysis was carried out in the same panel of 9 cell lines following 48 and 72 hours of incubation with the highest concentration of PrAg/LF used. There was no difference in cell cycle status between control cells and cells treated with PrAg/LF for the majority of cell lines at any time point. However, cell cycle arrest at the G_0/G_1 phase was seen in 2 cell lines, the HT-29 and SW948 following treatment with PrAg/LF at both 48 and 72 hours of incubation (Figure 3.3). In the HT-29 cell line, the fraction of G_0/G_1 phase increased by 23.7% following treatment with a corresponding decrease in the fraction of cells in the G2/M and S phases. This shows that the HT-29 cell line may express very low levels of anthrax toxin receptors allowing entry of LF to an extent that causes cell cycle arrest rather than cytotoxicity. Also in the SW948 cell line, the fraction of G_0/G_1 phase increased by 11.2 % following treatment accompanied by a corresponding decrease in the fraction of cells in G2/M and S phases (Figure 3.3). These results indicate that the LF-mediated inhibition of the MAPK pathway has limited cytostatic effects in CRC cell lines. This also further confirms the resistance of CRC cell line to the inhibition of MAPK pathway via PrAg/LF.

a)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M1
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M5
16.0%
M3
1.7%
Count
FL2-A
0
500,000
1,000,000
1,777,971
A01 Caco-2 PALF
Gate: (R1 in all)
M4
68.5%
M1
9.1%
M5
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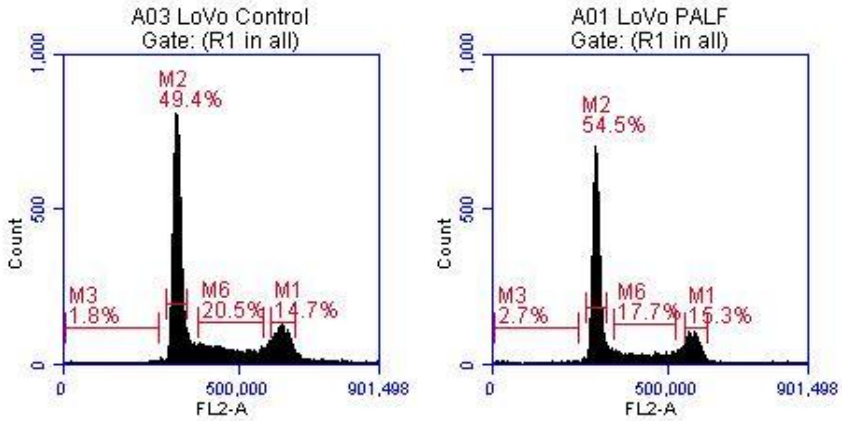
M3
2.4%
Count
FL2-A
0
500,000
1,000,000
1,777,971

b)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M3
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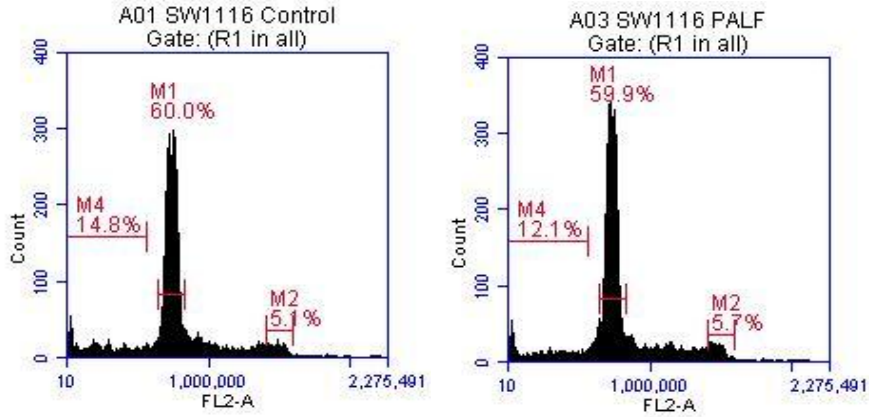
M6
20.5%
M1
14.7%
Count
FL2-A
0
500,000
901,498
A01 LoVo PALF
Gate: (R1 in all)
M2
54.5%
M3
2.7%
M6
17.7%
M1
15.3%
Count
FL2-A
0
500,000
901,498

c)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M4
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M2
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Count
FL2-A
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1,000,000
2,275,491
A03 SW1116 PALF
Gate: (R1 in all)
M1
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M4
12.1%
M2
5.7%
Count
FL2-A
10
1,000,000
2,275,491

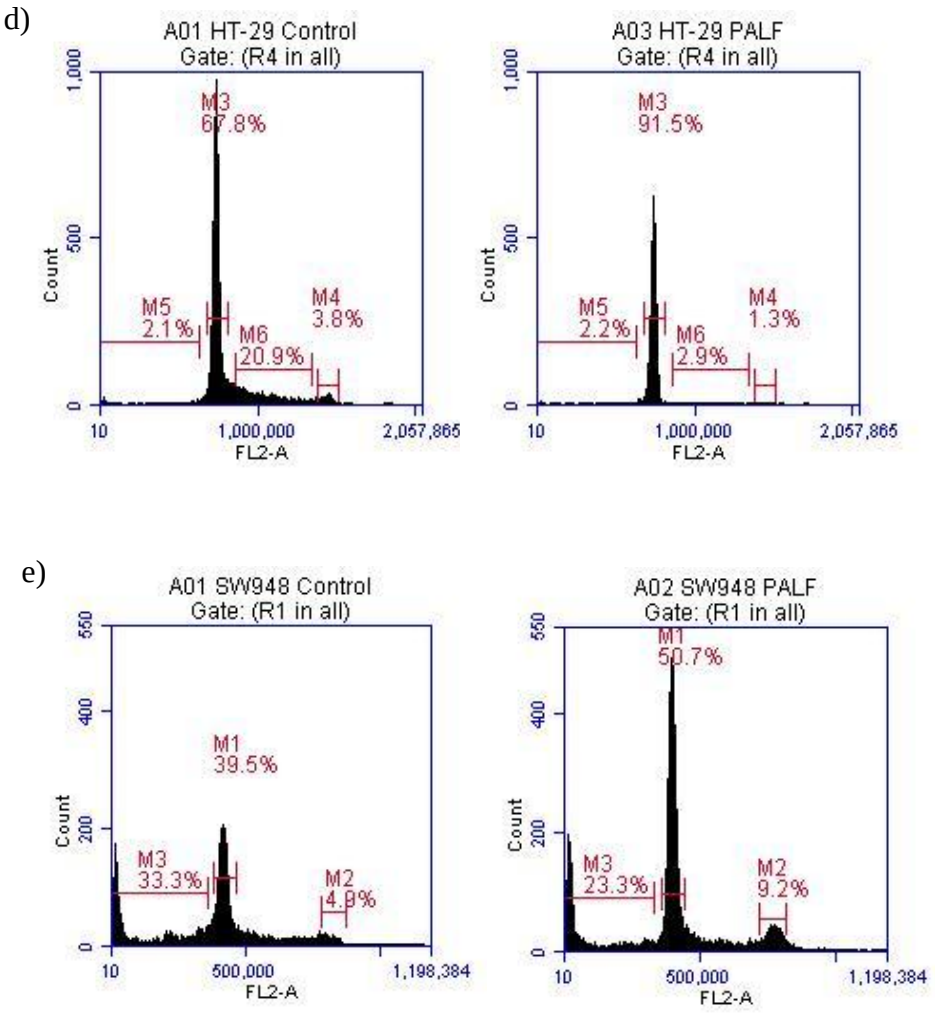
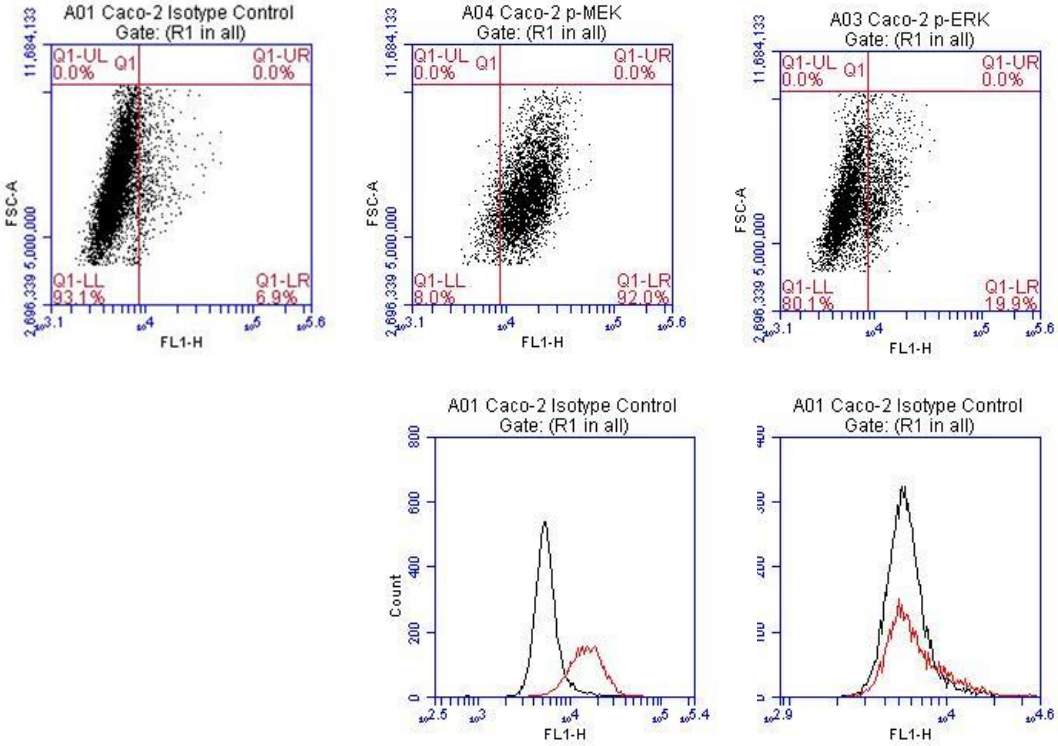


Figure 3.3. Cell cycle analysis of human CRC cell lines following treatment with PrAg/LF. Left panel represent control cells and right panel represent cells treated with 10^4pM for 48 hrs. Cells are gated on width versus forward scatter (R1/R2). Cells in G0/G1 are gated M1, G2/M are gated M2 and pre-G0/G1 (dead) are gated M3. No cell cycle arrest was shown in most cell lines, Caco-2 (a), LoVo (b), SW1116 (c) upon treatment with PrAg/LF for 48 hrs. A Small portion of cells exhibited cell cycle arrest at G0/G1 phase in HT-29 (d) and SW948 (e).

3.3. Analysis of MAPK activation

In order to understand the underlying mechanisms of the resistance of CRC cells to the inhibition of the MAPK pathway, we investigated the activation level of this pathway in CRC cell lines by determining the phosphorylation levels of its ERK1/2 and MEK1/2 components using the intracellular staining technique on flow cytometry. We chose to focus on the ERK1/2 branch of the pathway because in previous studies we had demonstrated that the cytotoxic effects of LF were mediated through the inhibition of this branch of the Pathway. The majority of cell lines were either positive or slightly positive for the presence of phosphorylated MEK1/2 except For HT-29, SW948 and T-84, which were clearly negative for phospho-MEK1/2. Remarkably, no phosphorylated ERK1/2 was detected in any of the CRC cell lines tested except in a relatively small proportion of cells of CaCo-2 and SK-Co-1, not involving more than 20% of the total cell population (Figure 3.4) (Table 3.2). This indicates that the MAPK pathway is not fully active in our CRC cell lines panel, and hence its inhibition using the fusion toxin PrAg/LF or the MEK1/2 specific-inhibitor would not affect cell proliferation or cell viability. It also indicates that phospho-ERK1/2 is the most important indicator of the activation of the MAPK pathway and subsequent sensitivity to its inhibition. This is in line with our findings in other tumor types including AML, in which sensitivity to the LF-mediated inhibition of the MAPK pathway depended on phosphor-ERK1/2 levels.

a)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Q1-LL 93.1%
Q1-LR 6.9%
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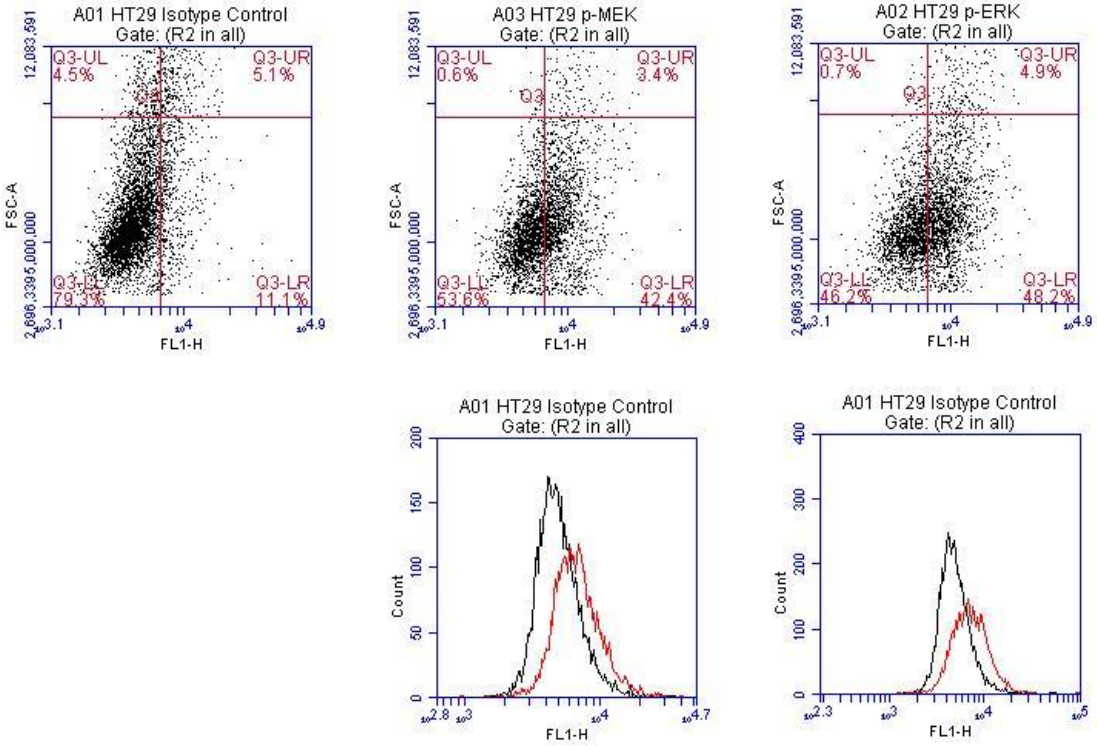
Gate: (R1 in all)
Q1-UL 0.0%
Q1-UR 0.0%
Q1-LL 8.0%
Q1-LR 92.0%
A03 Caco-2 p-ERK
Gate: (R1 in all)
Q1-UL 0.0%
Q1-UR 0.0%
Q1-LL 80.1%
Q1-LR 19.9%
FSC-A
FL1-H
A01 Caco-2 Isotype Control
Gate: (R1 in all)
A01 Caco-2 Isotype Control
Gate: (R1 in all)
Count
FL1-H

b)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Q3-LR 11.1%
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Gate: (R2 in all)
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Q3-UR 3.4%
Q3-LL 53.6%
Q3-LR 42.4%
A02 HT29 p-ERK
Gate: (R2 in all)
Q3-UL 0.7%
Q3-UR 4.9%
Q3-LL 46.2%
Q3-LR 48.2%
FSC-A
FL1-H
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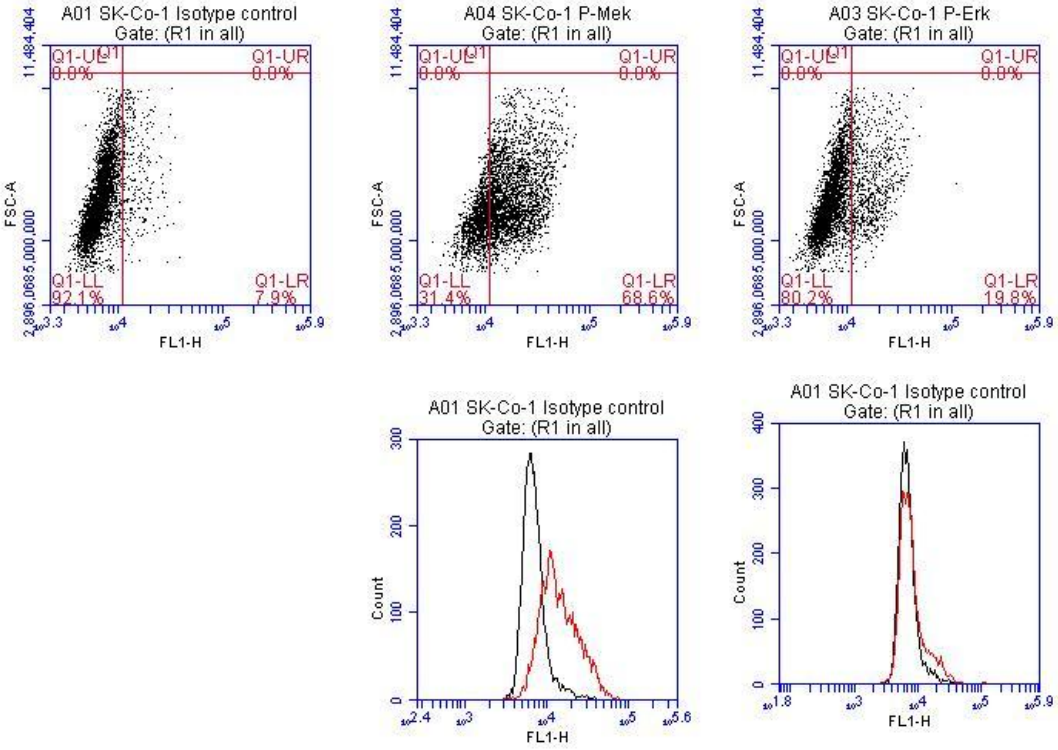
Gate: (R2 in all)
A01 HT29 Isotype Control
Gate: (R2 in all)
Count
FL1-H

c)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Q1-LR 7.9%
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Gate: (R1 in all)
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Q1-UR 0.0%
Q1-LL 31.4%
Q1-LR 68.6%
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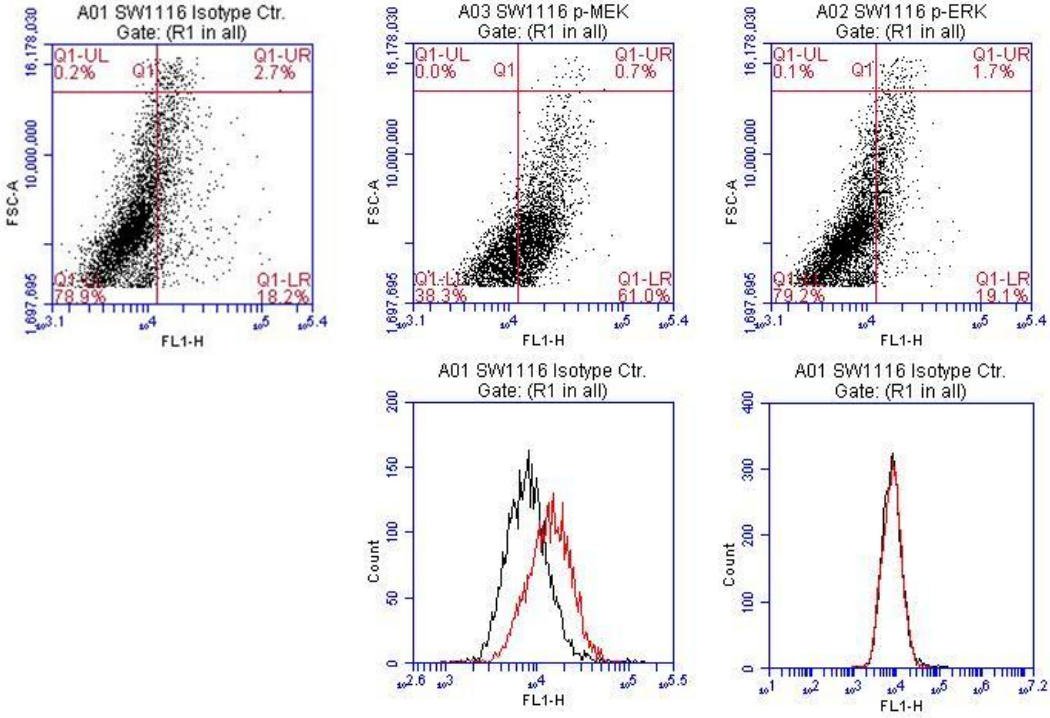
Gate: (R1 in all)
Q1-UL 0.0%
Q1-UR 0.0%
Q1-LL 80.2%
Q1-LR 19.8%
FSC-A
FL1-H
A01 SK-Co-1 Isotype control
Gate: (R1 in all)
A01 SK-Co-1 Isotype control
Gate: (R1 in all)
Count
FL1-H

d)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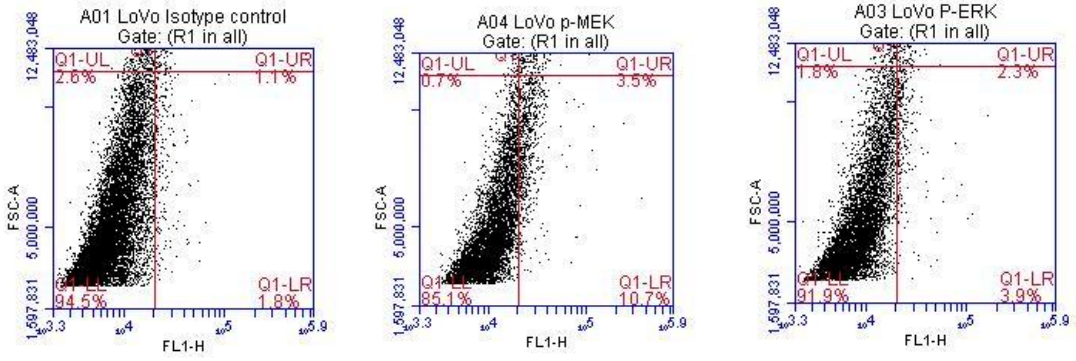
Q1-UL 0.0%
Q1-UR 0.7%
Q1-LR 61.0%
A02 SW1116 p-ERK
Gate: (R1 in all)
Q1-UL 0.1%
Q1-UR 1.7%
Q1-LR 19.1%
FSC-A
FL1-H
A01 SW1116 Isotype Ctr.
Gate: (R1 in all)
A01 SW1116 Isotype Ctr.
Gate: (R1 in all)
Count
FL1-H

e)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Q1-LL
94.5%
Q1-LR
1.8%
A04 LoVo p-MEK
Gate: (R1 in all)
Q1-UL
0.7%
Q1-UR
3.5%
Q1-LL
85.1%
Q1-LR
10.7%
A03 LoVo P-ERK
Gate: (R1 in all)
Q1-UL
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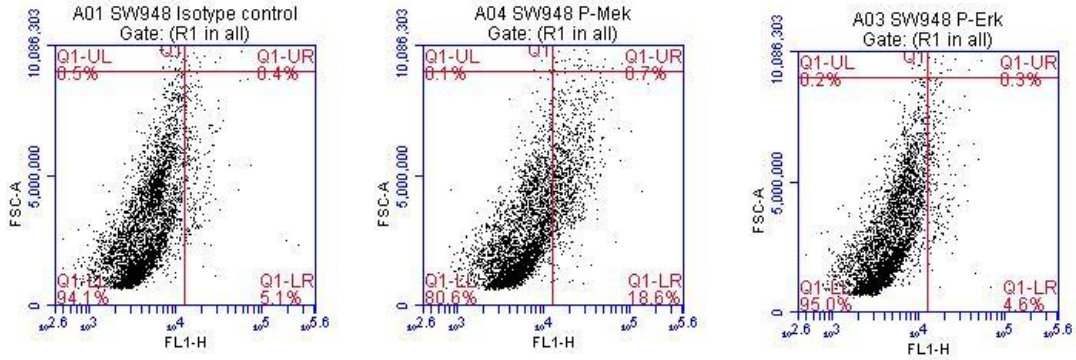
Q1-LR
3.9%
FSC-A
FL1-H

f)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Q1-LL
94.1%
Q1-LR
5.1%
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Gate: (R1 in all)
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Q1-UR
0.7%
Q1-LL
80.6%
Q1-LR
18.6%
A03 SW948 P-Erk
Gate: (R1 in all)
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Q1-UR
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Q1-LR
4.6%
FSC-A
FL1-H

g)

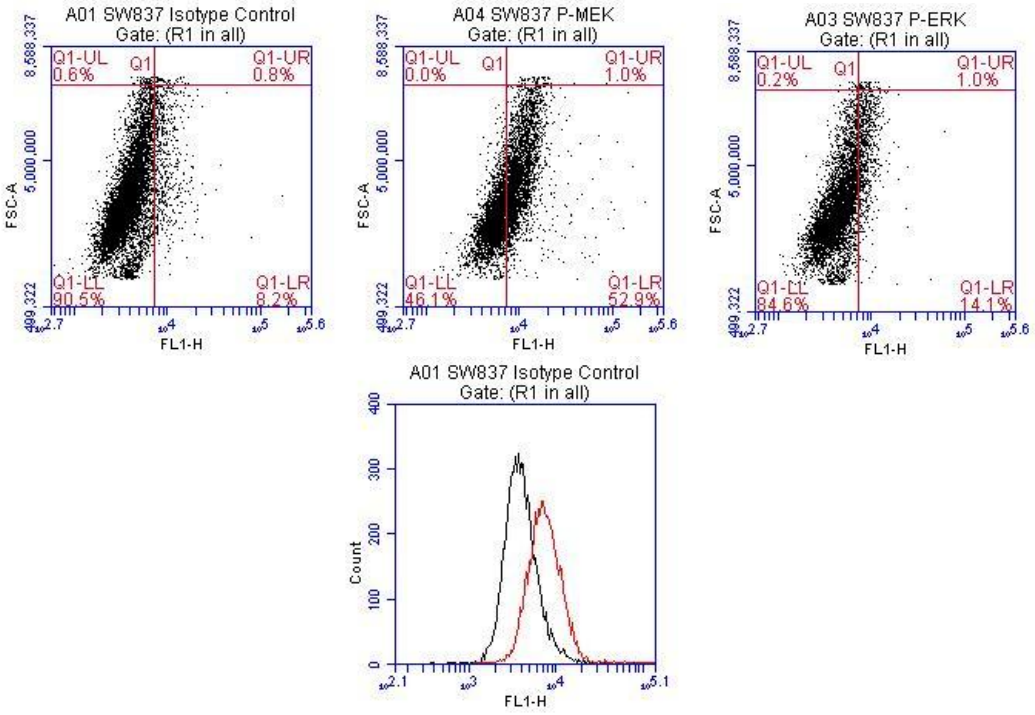


Figure 3.4. Analysis of MAPK activation. Intracellular staining of p-MEK1/2 and p-ERK1/2 through flow cytometry. High levels of active p-MEK were associated with the absence or very low levels of p-ERK in all tested CRC cell lines. Level of activation was determined according to the ratio of fluorescence intensity (RFI) of cells stained for phospho-MEK 1/2 or phospho-ERK1/2 (red) and cells incubated with the isotype control (black)., RFI> 2 was considered positive, RFI> 1.5 was considered slightly positive and RFI <1.5 was considered negative.

3.4. Inhibition of PI3K pathway

To determine whether CRC cell lines that are resistant to the inhibition of MAPK pathway could be sensitive to the inhibition of the PI3K/Akt pathway or whether a synergistic effect can be achieved by simultaneously inhibiting both the MAPK and PI3K pathways. We tested the sensitivity of a subset of CRC cell lines (SW837, T84, SK-Co-1 and SW1116) to the small molecular weight PI3K inhibitor LY294002, alone and in combination with PrAg/LF. None of the CRC cell lines tested were sensitive to LY294002, except SW837 which showed limited sensitivity to the inhibition of the PI3K/Akt pathway with an IC50 of approximately 11 micromolar (Figure 3.5). In addition, no additive effects were observed when LY294002 was used in combination with PrAg/LF, indicating resistance of the majority of CRC cell lines to the concomitant inhibition of both the MAPK and PI3K/Akt pathways (Figure 3.5).

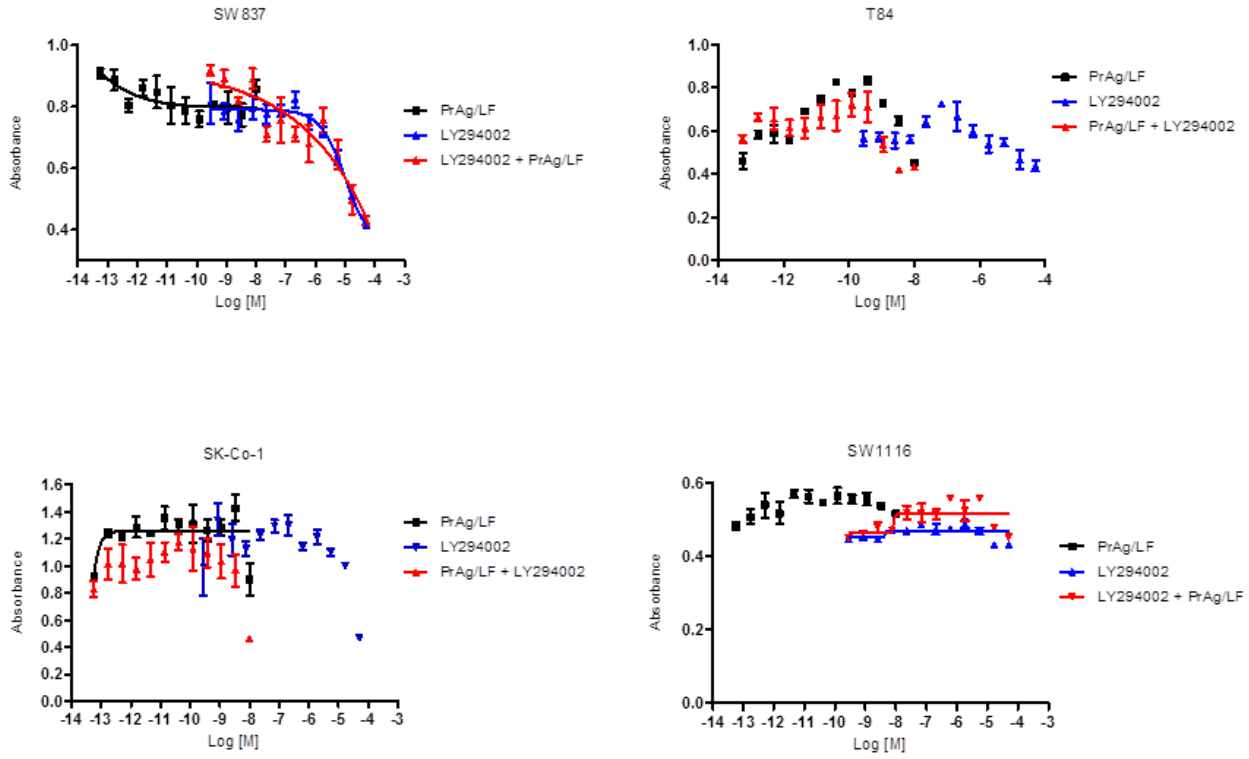
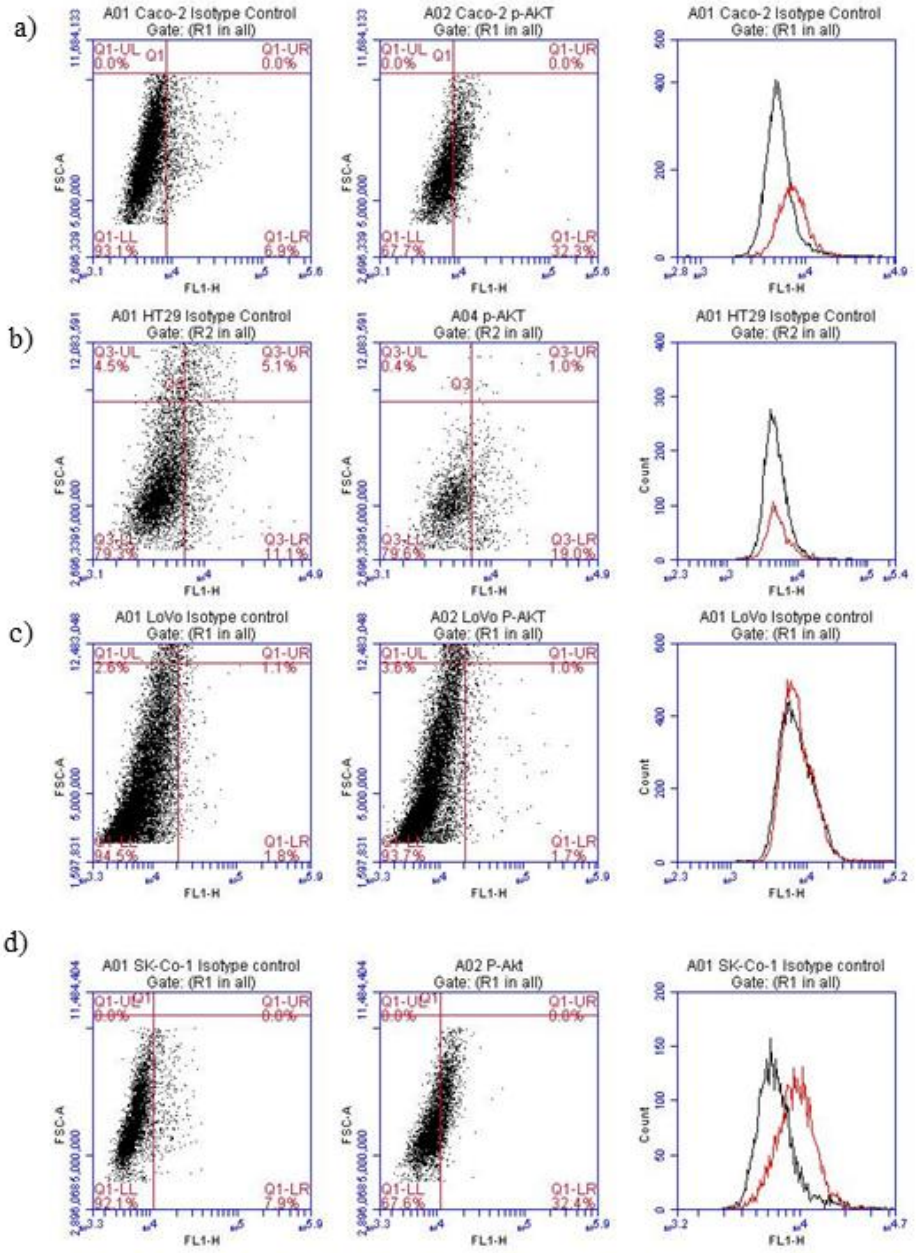


Figure 3.5. Sensitivity of cell lines to the inhibition of PI3K pathway through LY294002. None of the tested CRC cell lines were sensitive to LY294002, except SW837 which showed limited sensitivity to LY294002 with an IC50 of approximately 11 micromolar. Also, no additive effects were observed when using a combination of LY294002 andPrAg/LF.

3.5. Analysis of PI3K pathway activation

In order to investigate the underlying molecular mechanisms of the resistance of CRC cells to the inhibition of the PI3K/Akt pathway, we determined the activation level of this pathway in CRC cell lines by determining the phosphorylation levels of its AKT components using intracellular staining on flow cytometry. None of the cell lines tested were positive for p-AKT (Figure 3.6) indicating the lack of activation of this pathway in CRC. This also explains the resistance of CRC cell lines to the inhibition of PI3K pathway using the specific inhibitor LY294002. The fact that the PI3K pathway is not active in the CRC cell line panel corresponds to the fact that its inhibition does not affect cell viability (Figure 3.6).


a)
A01 Caco-2 Isotype Control
Gate: (R1 in all)
Q1-UL 0.0%
Q1-UR 0.0%
Q1-LL 93.1%
Q1-LR 6.9%
A02 Caco-2 p-AKT
Gate: (R1 in all)
Q1-UL 0.0%
Q1-UR 0.0%
Q1-LL 67.7%
Q1-LR 32.3%
A01 Caco-2 Isotype Control
Gate: (R1 in all)
RFI = 1.34
b)
A01 HT29 Isotype Control
Gate: (R2 in all)
Q3-UL 4.5%
Q3-UR 5.1%
Q3-LL 79.3%
Q3-LR 11.1%
A04 p-AKT
Gate: (R2 in all)
Q3-UL 0.4%
Q3-UR 1.0%
Q3-LL 79.6%
Q3-LR 19.0%
A01 HT29 Isotype Control
Gate: (R2 in all)
RFI = 1.06
c)
A01 LoVo Isotype control
Gate: (R1 in all)
Q1-UL 2.6%
Q1-UR 1.1%
Q1-LL 94.5%
Q1-LR 1.8%
A02 LoVo P-AKT
Gate: (R1 in all)
Q1-UL 3.6%
Q1-UR 1.0%
Q1-LL 93.7%
Q1-LR 1.7%
A01 LoVo Isotype control
Gate: (R1 in all)
RFI = 1.03
d)
A01 SK-Co-1 Isotype control
Gate: (R1 in all)
Q1-UL 0.0%
Q1-UR 0.0%
Q1-LL 92.1%
Q1-LR 7.9%
A02 P-Akt
Gate: (R1 in all)
Q1-UL 0.0%
Q1-UR 0.0%
Q1-LL 67.6%
Q1-LR 32.4%
A01 SK-Co-1 Isotype control
Gate: (R1 in all)
RFI = 1.32
FSC-A
FL1-H
Count

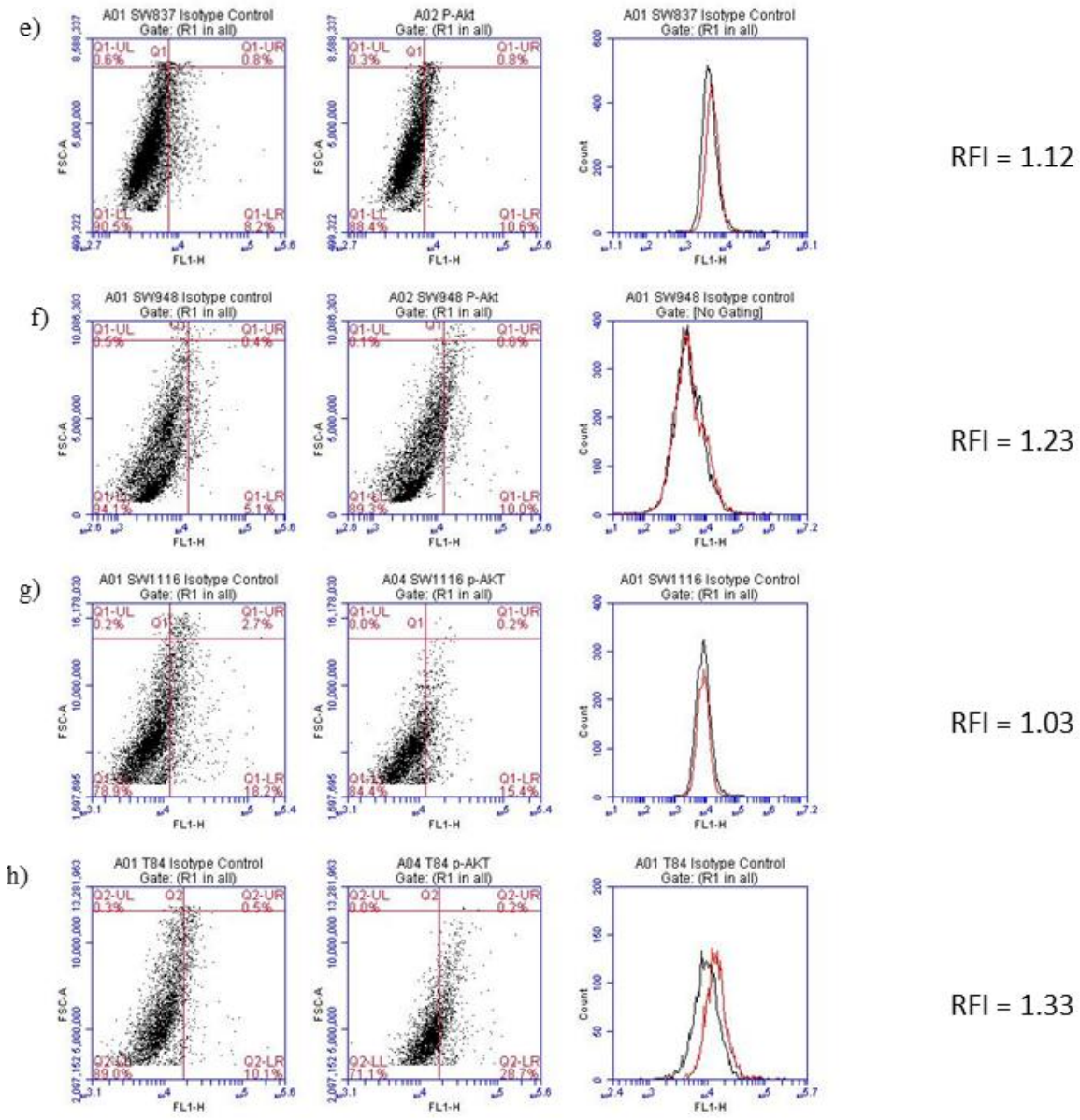
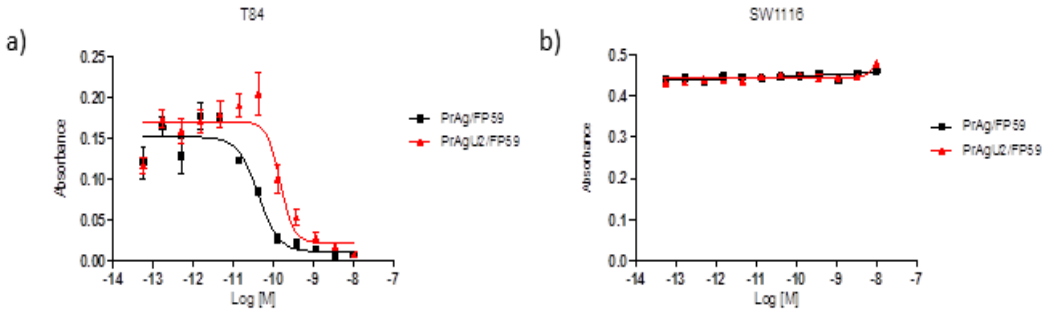


Figure 3.6. Activation status of PI3K pathway measured by level of p-AKT using intracellular staining on flow cytometry. The left panels represent cells stained with isotopic control and the right panels cells stained with anti phospho-Akt antibody. Cells were gated on width versus forward scatter. Positivity was determined by RFI which was less than 1.5 for all the cell lines indicating no p-AKT found, hence PI3K pathway is not active.

3.6. Cytotoxicity of PrAgU2/FP59

In order to assess the possibility of targeting the uPA/uPAR protease system alone, independently of the MAPK pathway, we tested the sensitivity of CRC cells to PrAgU2/FP59, a urokinase activated, MAPK-independent version of the toxin (Figure 3.7). As expected, the 4 cell lines that did not express anthrax toxin receptors were not sensitive to PrAgU2/FP59 due to their inability to bind the toxin. However, out of the 5 remaining cell lines that did express anthrax toxin receptors, 4 were sensitive to the urokinase-activated PrAgU2/FP59 (SNU-c1, T84, LoVo and SK-Co-1) with IC_{50} values ranging from 98 to 930 pM (Table 3.2, Figure 3.7). These results demonstrate the possibility of targeting the urokinase plasminogen activator protease system in CRC cell lines independently from targeting MAPK pathway.



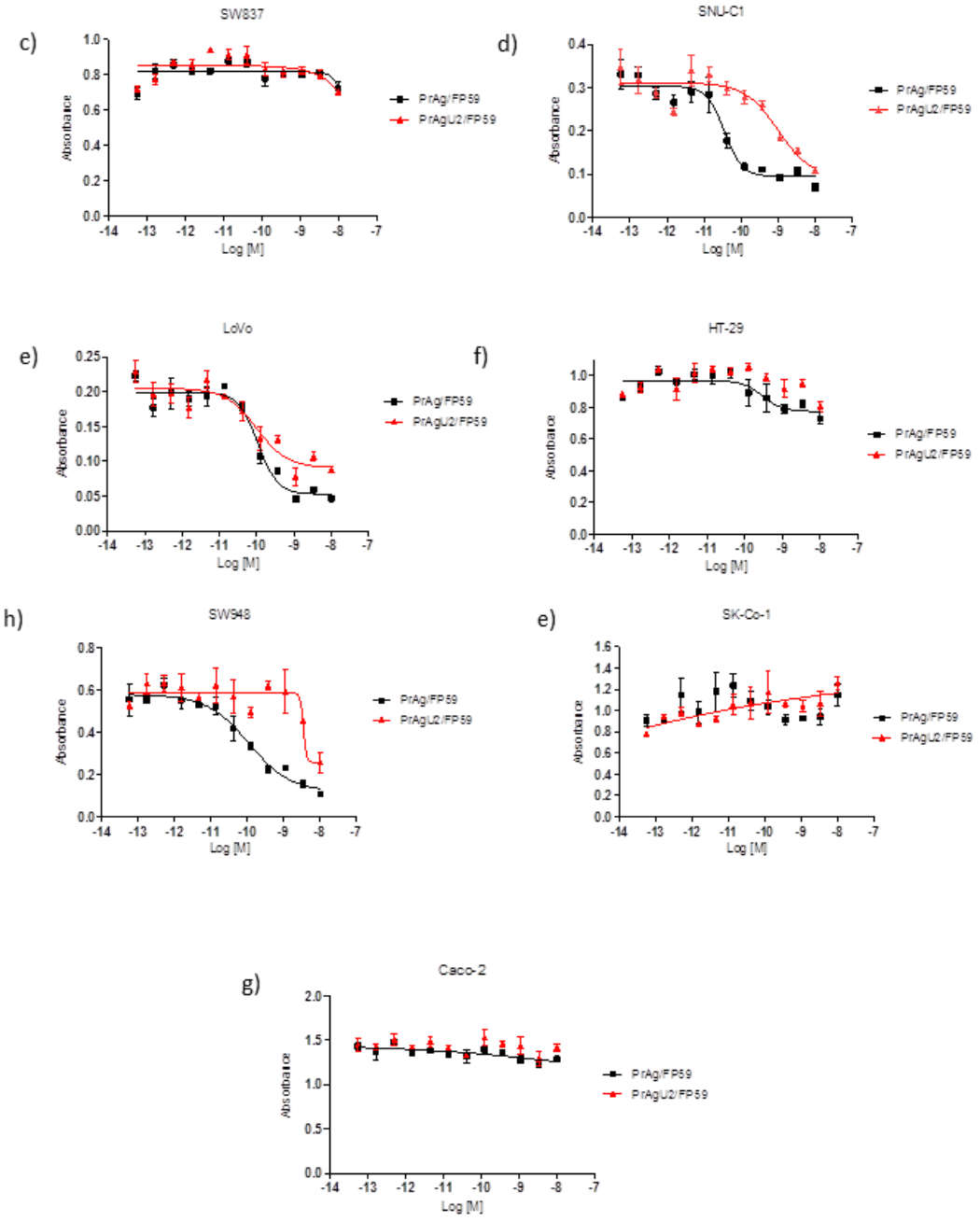
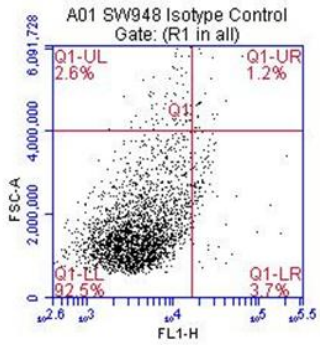
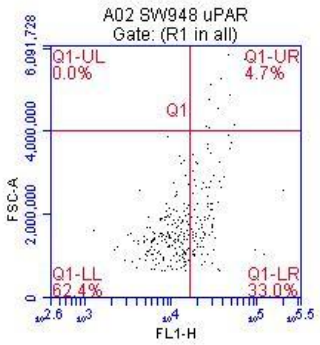


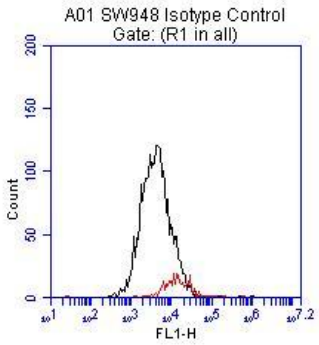
Figure 3.7 . Cytotoxic effect of PrAgU2/FP59 on CRC cell lines.4cell lines were sensitive to the urokinase-activated PrAgU2/FP59 (SNU-c1, T84, LoVo and SK-Co-1) with IC_{50} values ranging from 98 to 930 pM. While 4 cell lines (HT-29. SW837, Caco-2 and SW1116) were not sensitive to PrAgU2/FP59 as they were not expressing anthrax toxin receptors and thus cannot bind the toxin.

3.7. uPAR expression

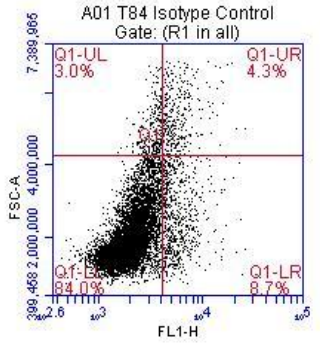
In order to determine the underlying mechanisms of the cytotoxicity of PrAgU2/FP59 to CRC cells, we assessed the expression levels of the urokinase plasminogen activator receptor (uPAR) through cell surface staining on flow cytometry. Positive uPAR expression was revealed by a ratio of fluorescence intensity of 2-folds or more for the uPAR stained cells in comparison to isotopic control cells. RFI between 1.5 and 2.00 was considered slightly positive to the uPAR expression, while RFI <1.5 was considered negative. All but two of the cell lines tested exhibited high levels of uPAR expression with a ratio of fluorescence intensity (RFI) ranging from 2.33 to 17.02. The 2 remaining cell lines were slightly positive (SW837, RFI = 1.59) and negative (SW1116, RFI = 1.16) for the expression of uPAR (Figure 3.8, Table 3.2). Results indicate a strong correlation between the uPAR expression and sensitivity to targeting the urokinase plasminogen activator system in CRC cells using PrAgU2/FP59. This demonstrates that CRC cell lines, for the most part, express high levels of uPAR rendering this prognostic marker a potential target for therapy of CRC.

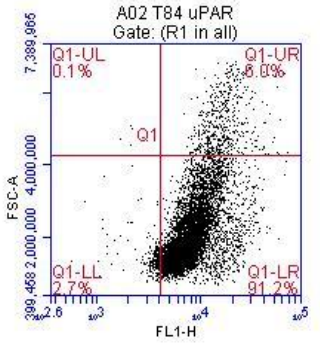


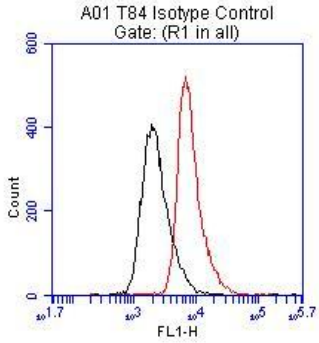




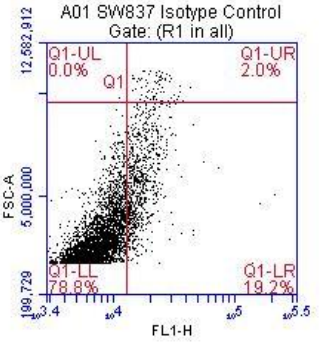
RFI = 3.07

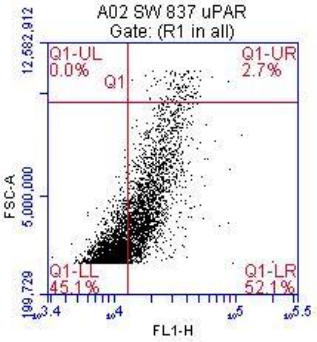


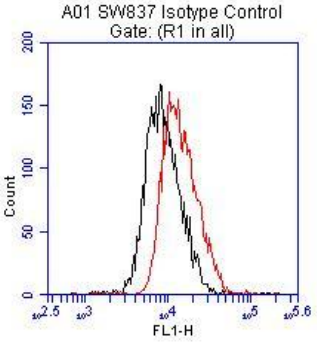




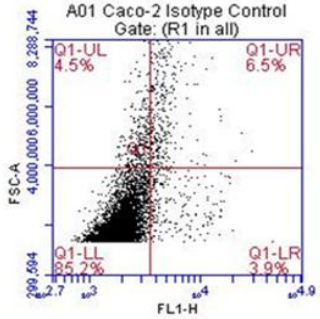
RFI = 3.59

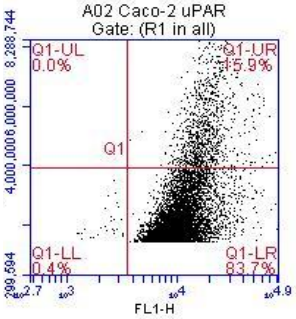


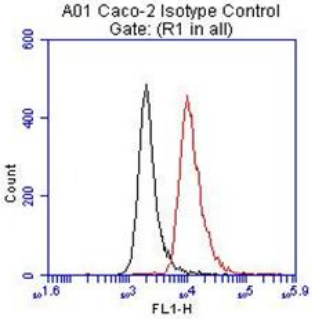




RFI = 1.59







RFI = 5.73

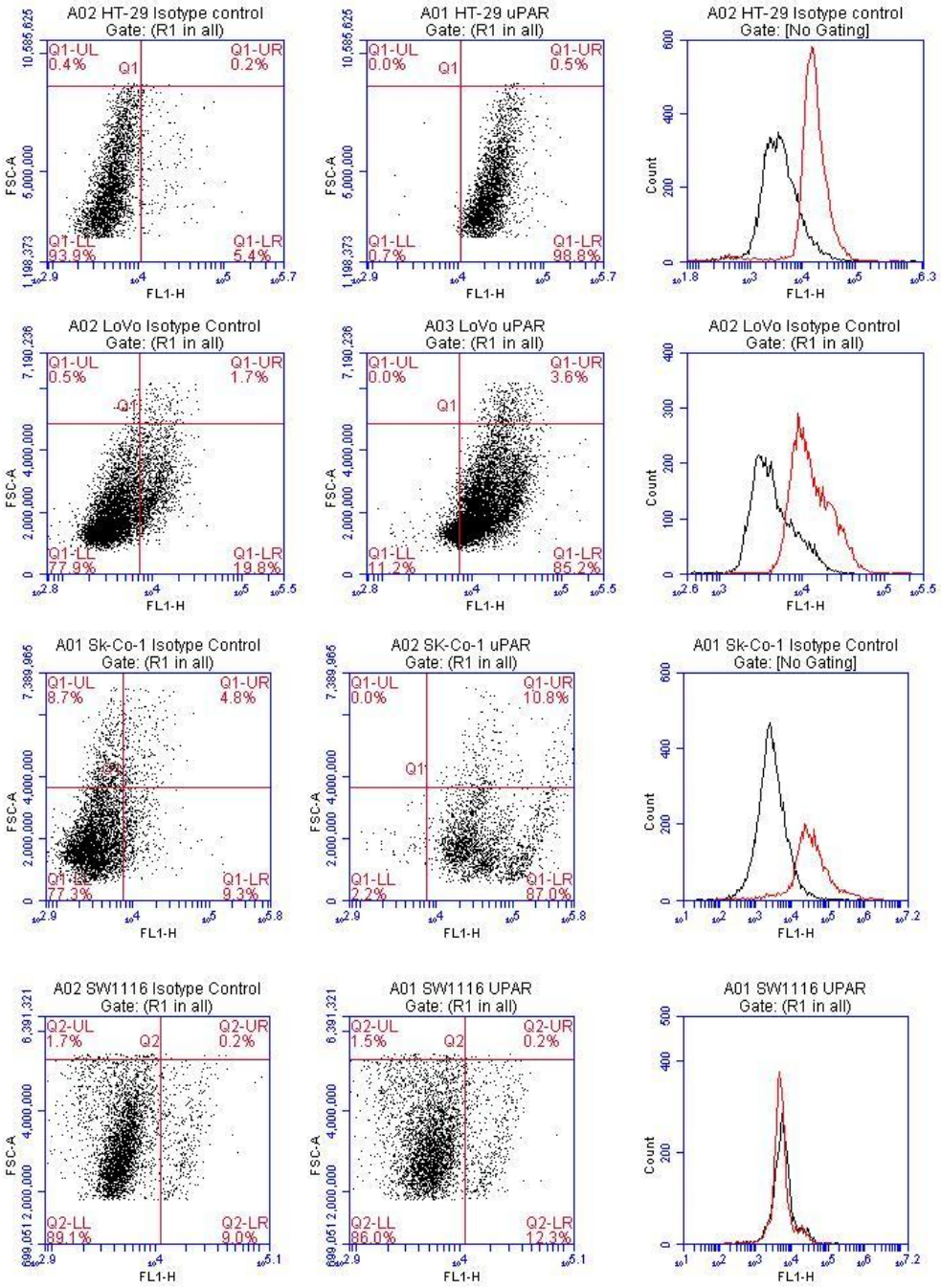


Figure 3.8. uPAR expression in CRC cell lines. SW1116 was considered negative for the expression of uPAR with RFI = 1.16, SW837 was considered slightly positive with RFI = 1.59. all the other cell lines exhibited high levels of uPAR expression with a ratio of fluorescence intensity (RFI) ranging from 2.33 to 17.02.

Table 3.2. Sensitivity of CRC cell lines to PrAg/LF and PrAg/FP59 and levels of MAPK activation represented by phospho-MEK 1/2 and phospho-ERK 1/2 levels, In addition to their expression of uPA receptors.

Cell line	PrAg/LF (IC_{50}, pmol/L)[#]	Phospho-MEK1/2 (RFI)	Phospho-ERK1/2 (RFI)	PrAgU2/FP59 (IC_{50}, pmol/L)	uPAR (RFI)[*]
SNU-c1	71	NA	NA	930	N/A
T84	3950	1.12 (-)	1.33 (-)	146	3.59 (+)
LoVo	1641	1.56 (+/-)	1.21 (-)	98	2.87 (+)
Sk-Co-1	8120	2.27 (+)	1.24 (-)	NA	17.02 (+)
SW948	3041	1.45 (-)	0.92 (-)	3449	N/A
HT-29	>10000	1.37 (-)	1.48 (-)	>10000	2.33 (+)
SW837	>10000	2.14 (+)	1.13 (-)	>10000	1.59 (+/-)
Caco-2	>10000	2.81 (+)	1.17 (-)	>10000	5.73 (+)
SW1116	>10000	1.69 (+)	0.96 (-)	>10000	1.16 (-)

N/A: Not available. [*] The (+) and (-) signs indicate positive and negative RFI for uPAR expression, respectively. [#] Same data as the one presented in the second column of Table 3.1.

CHAPTER FOUR

DISCUSSION

Oncogenic mutations in the Mitogen-activated protein kinase pathway have been identified as one of the most strongly associated gene markers to colorectal cancer CRC, especially mutations in the RAs–RAf–MEK1/2–ERK1/2 pathway which have been found in around 20-25 % of all human cancers. This suggests that the Ras–RAf–MEK1/2–ERK1/2 axis is crucial for survival of this tumor. Moreover, studies have shown that CRC tissues have excessive expression of the Urokinase Plasminogen Activator protease system which is one important hallmark of invasive and metastatic cancer. In this view, we attempted in our study to target these 2 major hallmarks of CRC, the MAPK pathway and the uPA/uPAR system using the dual-selective Urokinase-activated, recombinant anthrax lethal toxin PrAgU2/LF in comparison to the single targeting moiety (Recombinant anthrax lethal toxin - PrAg/LF). The recombinant anthrax lethal toxin (PrAg/LF) is a targeted protein therapeutic that would bind to all cells and get activated upon cleavage by furin and lead to the translocation of LF into the cytosol. Once in the cytosol, LF inhibits all branches of the MAPK pathway via cleavage and inactivation of MEKs, and thus it has been of a great interest for tumor-selective cancer therapy. On the other hand , our main interest was to test the Urokinase-activated, recombinant anthrax lethal toxin PrAgU2/LF which is a dual-selective fusion toxin where the binding moiety can only be activated upon binding to uPA expressing

cells, and leading then to LF translocation into the cytosol where it inhibits the MAPK pathway resulting in cell death.

A panel of 9 human CRC cell lines was employed in this study. Most of them are K-Ras mutants and therefore, we hypothesized would be dependent on the MAPK pathway for survival. PrAg/LF showed no cytotoxic effect on all but one of the 9 CRC cell lines tested. This was very surprising given the fact that the majority of cell lines were k-Ras mutant and were expected to have an active MAPK pathway. Therefore, and as expected, none of the cell lines were sensitive to the urokinase-activated PrAgU2/LF. Resistance to the PrAg/LF and PrAgU2/LF can be explained either by the absence of anthrax lethal toxin receptors or by these cells being MAPK-independent for survival. Thus, we used PrAg/FP59 fusion toxin as a control to test for presence or absence of the anthrax lethal toxin receptors. PrAg/FP59 is a recombinant protein composed of the PrAg binding domain of LF fused to the catalytic domain of *Pseudomonas aeruginosa* exotoxin A. Binding of FP59 to PrAg and its translocation into the cytosol are identical to those of LF, however, it does not target the MAPK pathway but rather ADP-ribosylates elongation factor 2 leading to inhibition of protein synthesis and cell death. Five Cell lines were sensitive to PrAg/FP59 indicating that they express anthrax toxin receptors on their surface. However, the remaining 4 cell lines were resistant to PrAg/FP59 indicating the absence of anthrax toxin receptors on the cell surface and the subsequent inability to bind, activate and internalize the toxin.

Moreover, we used the U0126 small molecule in order to investigate the cells response to MEK1/2 inhibition. None of the cell lines tested were sensitive to the inhibition of MAPK pathway via U0126. This result has further confirmed the lack of

sensitivity of our CRC panel of cell lines to MAPK pathway inactivation neither by LF-mediated inhibition nor by the U0126 MEK1/2 specific inhibitor. In addition, marginal cell cycle arrest was observed in some of the CRC cell lines tested indicating that, in addition to the lack of any significant cytotoxic effect, LF also lacks any cytostatic effect in CRC cells. This was surprising and differed from our experience in other tumor types such as melanoma and acute myeloid leukemia (AML) where a majority of cells were found to be dependent on the MAPK pathway for survival. Investigating the MAPK activation levels revealed a high levels of phosphorylated MEK 1/2, but surprisingly no active phosphorylated ERK 1/2. These results indicate that the MAPK pathway is not active, despite the presence of phospho-MEK1/2 since ERK1/2, the downstream effector of the MAPK pathway, is not active. Thus, it can be deduced that these cells are not dependent on the MAPK pathway for survival, even though they are Ras+ mutants. Hence, targeting the MAPK pathway would not sensitize the cells and would cause no LF-mediated cytotoxicity. This data is consistent with Abi-Habib et al. 2005, where the study showed that constitutive activation of the mitogen-activated protein kinase pathway associated with BRAF mutation status are actually sensitive to the anthrax lethal toxin, whereas ras+ mutants were resistant to the fusion toxin. None of the CRC cell lines were significantly sensitive to the inhibition of PI3K pathway using the LY924002 PI3 kinase inhibitor. Also, none were sensitive to the co-treatment with both PrAg/LF and LY924002. Activation level of the PI3 kinase pathway was checked by investigating the phosphorylated levels of Akt component. No active phospho-Akt was detected on flow cytometry for any of the 8 cell lines tested, suggesting that CRC cell lines are also independent from PI3 kinase pathway for survival, and no synergistic

effect could be achieved by the inhibition of both the MAPK and PI3K pathway at the same time.

In order to assess the possibility of targeting the uPA/uPAR system alone, we tested the sensitivity of CRC cells to PrAgU2/FP59, a urokinase activated, MAPK-independent version of the toxin. PrAgU2/FP59 gets activated only in cells expressing uPA and leads to the translocation of FP59 into the cytosol where the latter acts by the inhibition of protein synthesis resulting in cell death. The vast majority of cell lines showed sensitivity to PrAgU2/FP59. Moreover, we determined the uPAR expression level on all 8 cell lines by uPAR cell surface staining on flow cytometry. Most of the cell lines were positive for uPAR expression suggesting a strong correlation between sensitivity to PrAgU2/FP59 and uPAR expression. These results indicate the possibility for targeting this protease system in CRC, besides considering the uPAR expression as a marker for sensitivity to the uPA/uPAR system targeting.

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