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Prediction of Proapoptotic Anticancer Therapeutic Response Based on Visualization of Death Ligand-Receptor Interaction and Specific Marker of Cellular Proliferation

Abstract

Emerging targeted therapeutics hold great promise for the treatment of human cancer. However there are still challenges for selecting patients that most likely will benefit from targeted drugs. One of the major limitations of classical imaging methods is the significant delay to provide quantifiable and objective evidence of response to cancer therapy. Molecular imaging may be useful in targeted drug development by assessing the target expression and drug-target interaction, and predicting therapeutic response in both preclinical and clinical settings. The apoptosis pathway triggered by the Tumor Necrosis Factor (TNF)-Related Apoptosis-Inducing Ligand (TRAIL) receptors is a potential target for therapeutic intervention. TRAIL and its proapoptotic receptor agonistic monoclonal antibodies are being developed as targeted therapeutics in the treatment of human cancer. It is our hypothesis that visualization of proapoptotic receptors and binding of their agonists to proapoptotic receptors can noninvasively predict proapoptotic response if the pathway is intact. Hence the objective of this work is to develop efficient multimodality molecular imaging methods to predict proapoptotic anticancer therapy response before or at the very early stage of treatment. Towards this goal, we have labeled proapoptotic receptor agonists (PARAs) with near-infrared (NIR) fluorescent dyes to image PARAs binding to their targets expressed on the cell surface in cultured cells and in human tumor xenografts grown subcutaneously in immunodeficient mice. Both in vitro and in vivo studies demonstrated that imaging PARAs binding to their targets was well correlated with proapoptotic anticancer therapeutic response when TRAIL signaling pathway was intact. To pursue a more general molecular imaging marker that can predict anticancer therapeutic response even when the signaling pathway is impaired, we explored a novel radiotracer for positron emission tomography (PET) imaging [(18)F]-3'-fluoro-3'-deoxy-L-thymidine ([(18)F]-FLT), an analogue of thymidine and a specific marker of DNA replication and cellular proliferation. Our results suggested that early changes in [(18)F]-PET may not only predict the tumor histological response to anticancer therapeutics but also determine superiority of one treatment regimen over another. In summary our proof-of-concept studies show that multimodality molecular imaging will greatly aid in accelerating anticancer drug approval process and improving survival and response rates in hard-to-treat cancer.

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PREDICTION OF PROAPOPTOTIC ANTICANCER THERAPEUTIC RESPONSE BASED ON VISUALIZATION OF DEATH LIGAND-RECEPTOR INTERACTION AND SPECIFIC MARKER OF CELLULAR PROLIFERATION

Lanlan Zhou

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Requirements for the Degree of Doctor of Philosophy

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PREDICTION OF PROAPOPTOTIC ANTICANCER THERAPEUTIC RESPONSE BASED ON VISUALIZATION OF DEATH LIGAND-RECEPTOR INTERACTION AND SPECIFIC MARKER OF CELLULAR PROLIFERATION

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Lanlan Zhou

DEDICATION

To my family – I would not be where I am today without your unconditional love, inspiration, understanding, encouragement and support!

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ABSTRACT

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Lanlan Zhou

Supervisor: Wafik S. El-Deiry, M.D. Ph.D.

Emerging targeted therapeutics hold great promise for the treatment of human cancer. However there are still challenges for selecting patients that most likely will benefit from targeted drugs. One of the major limitations of classical imaging methods is the significant delay to provide quantifiable and objective evidence of response to cancer therapy. Molecular imaging may be useful in targeted drug development by assessing the target expression and drug-target interaction, and predicting therapeutic response in both preclinical and clinical settings. The apoptosis pathway triggered by the *T*umor *N*ecrosis *F*actor (*T*NF)-*R*elated *A*poptosis-*I*nducing *L*igand (TRAIL) receptors is a potential target for therapeutic intervention. TRAIL and its proapoptotic receptor agonistic monoclonal antibodies are being developed as targeted therapeutics in the treatment of human cancer. It is our hypothesis that visualization of proapoptotic receptors and binding of their agonists to proapoptotic receptors can noninvasively predict proapoptotic response if the pathway is intact. Hence the objective of this work is to develop efficient multimodality molecular imaging methods to predict proapoptotic anticancer therapy response before or

at the very early stage of treatment. Towards this goal, we have labeled proapoptotic receptor agonists (PARAs) with near-infrared (NIR) fluorescent dyes to image PARAs binding to their targets expressed on the cell surface in cultured cells and in human tumor xenografts grown subcutaneously in immunodeficient mice. Both in vitro and in vivo studies demonstrated that imaging PARAs binding to their targets was well correlated with proapoptotic anticancer therapeutic response when TRAIL signaling pathway was intact. To pursue a more general molecular imaging marker that can predict anticancer therapeutic response even when the signaling pathway is impaired, we explored a novel radiotracer for positron emission tomography (PET) imaging ¹⁸F-3'-fluoro-3'-deoxy-L*t*hymidine (18 F-FLT), an analogue of thymidine and a specific marker of DNA replication and cellular proliferation. Our results suggested that early changes in ¹⁸F-FLT-PET may not only predict the tumor histological response to anticancer therapeutics but also determine superiority of one treatment regimen over another. In summary our proof-ofconcept studies show that multimodality molecular imaging will greatly aid in accelerating anticancer drug approval process and improving survival and response rates in hard-to-treat cancer.

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CHAPTER 1

Introduction

1.1 Cancer and apoptosis

Cancer is the second most common cause of death in the US (American Cancer Society 2010). The 5-year relative survival rate for all cancers diagnosed between 1995 and 2001 was 65% (Parkin, Bray et al. 2005). Insufficient apoptosis can result in cancer. Apoptosis, an evolutionarily conserved mechanism of programmed cell death mediated principally by caspases, plays a crucial role in successful embryonic development and maintains normal cellular homeostasis in adult organisms (Kerr, Wyllie et al. 1972). The proapoptotic caspase cascade acts as the principal executioner in apoptosis - the initiators (caspases 2, 8, 9, and 10) activate the effectors (caspases 3, 6, and 7) to execute apoptosis. Cells undergo apoptosis through two separate but interlinked signaling mechanisms: the extrinsic pathway, activated by proapoptotic receptor signals at the cellular surface, and the intrinsic pathway, activated by mitochondrial signals within the cell. They converge through effector caspases (Lavrik, Golks et al. 2005).

The extrinsic pathway is initiated by members of the *t*umor *n*ecrosis *f*actor (TNF) superfamily as described in the section of the *t*umor *n*ecrosis *f*actor-*r*elated *a*poptosis*i*nducing *l*igand (TRAIL) signaling. The intrinsic pathway triggers apoptosis in response to loss of survival factors or severe cell stresses such as DNA damage. The intrinsic pathway involves the activation of the proapoptotic Bcl-2 proteins and the release of apoptogenic factors such as cytochrome c and SMAC/DIABLO from the mitochondria into the cytosol (Adams and Cory 1998; Green 2000; Hunt and Evan 2001). In the cytosol, cytochrome c binds the adaptor *a*poptotic *p*rotease-*a*ctivating *f*actor *1* (APAF-1) and the initiator caspase 9 to form the multimeric apoptosome (a complex comprising APAF-1, *d*eoxy*a*denosine *trip*hosphate (dATP), cytochrome *c* and caspase 9) to activate the initiator caspase-9. In turn, caspase-9 activates effector caspase-3, -6, and -7. SMAC/DIABLO promotes apoptosis by binding to *i*nhibitor of *a*poptosis *p*roteins (IAP) and preventing these factors from attenuating caspase activation (Du, Fang et al. 2000; Verhagen, Ekert et al. 2000). The intrinsic and extrinsic apoptosis signaling pathways communicate with each other. Caspase-8 has been shown to cleave the proapoptotic Bcl-2 family member Bid. The cleavage of Bid by caspase-8 and the translocation of truncated Bid to the mitochondria to promote cytochrome c release through interaction with Bax and Bak provide a plausible mechanistic link between the extrinsic and intrinsic pathways (Green 2000). This apparently amplifies the apoptotic signal following death receptor activation, and different cell types may be more reliant on this amplification pathway than others (Fulda, Meyer et al. 2001). Thereby activators of the intrinsic pathway can sensitize cells to extrinsic death ligands.

1.2 TRAIL signaling

TRAIL is a TNF family member. It is also designated as *Apo-2 l*igand (Apo-2L) because it is related most closely to Fas/Apo-1 ligand. TRAIL consists of 281 and 291 *a*mino *a*cids (AA) in the human and murine forms respectively with 65% AA identity. TRAIL is expressed at the cell surface with its carboxyl terminus exposed, indicating a type II transmembrane protein topology. The C-terminal extracellular region of TRAIL, AA 114-281, exhibits clear homology to other TNF family members, and is processed proteolytically to form a soluble homotrimeric molecule (Wiley, Schooley et al. 1995; Pitti, Marsters et al. 1996). The TRAIL gene is located on chromosome 3 at position 3q26 (Wiley, Schooley et al. 1995). The biological role of TRAIL is not fully understood yet. While TRAIL mRNA is constitutively expressed in a wide variety of normal tissues, most predominantly in spleen, lung and prostate, the expression of functional TRAIL protein appears to be rather restricted to immune cells, including T cells, NK cells, monocytes, dendritic cells and neutrophils. TRAIL plays important roles in inflammation, the immune response and tumor surveillance (Ashkenazi and Dixit 1999). TRAIL receptor deficient mice showed that metastasis to lymph nodes was significantly enhanced, indicating that TRAIL-R could inhibit metastasis. The possible mechanism is that detachment from the primary tumor can sensitize resistant TRAIL-R-expressing cancer cells to TRAIL (Grosse-Wilde, Voloshanenko et al. 2008).

Both full-length cell surface expressed TRAIL and picomolar concentrations of *r*ecombinant soluble TRAIL (rsTRAIL) rapidly induce apoptosis in a wide variety of transformed cell lines of diverse origin (Wiley, Schooley et al. 1995; Pitti, Marsters et al. 1996). Unlike conventional cancer therapeutics, TRAIL triggers tumor cell apoptosis independently of the p53 tumor suppressor gene, which frequently is inactivated in cancer. TRAIL offers promising therapeutic potential based on its ability to induce apoptosis in various cancer cell lines with little toxicity toward normal cells. Moreover, TRAIL displays single-agent activity and cooperates with chemotherapy or radiotherapy in a variety of tumor xenograft mouse models. Thus, TRAIL might be effective against tumors that have acquired resistance to conventional therapy, and could augment the efficacy of current treatment in a wide spectrum of cancers (Kelley and Ashkenazi 2004). The clinical utility of TNF and Fas has been hampered by toxicity to normal tissues. Intravenous TNF administration causes hypotension and a systemic inflammatory

syndrome that resembles septic shock due to strong activation of pro-inflammatory *n*uclear factor κ -B (NF- κ B). Injection of agonist anti-Fas antibodies induces hepatocyte apoptosis and lethal hepatic failure in mice (Feinberg, Kurzrock et al. 1988; Ogasawara, Watanabe-Fukunaga et al. 1993; Lejeune, Kroon et al. 2001; Hochedlinger, Wagner et al. 2002). TRAIL is still considered a promising anticancer agent due to a more acceptable toxicity profile. In preclinical models, it induces apoptosis in a wide range of tumor cells and xenografts, without causing toxicity to normal cells (Ashkenazi 2002). A combination treatment of TRAIL with ionizing irradiation or chemotherapeutic agents induces apoptosis in a highly synergistic manner, particularly in those cells that are otherwise resistant to the individual treatments. Furthermore, in contrast to its structural and functional homologues TNF and Fas-L, systemic exposure to TRAIL does not exert overt systemic toxicity in murine and primate models although concerns about a potential hepatotoxicity associated with nonoptimized preparations of TRAIL have been raised (Held and Schulze-Osthoff 2001; Kelley and Ashkenazi 2004; Ashkenazi, Holland et al. 2008). Currently, rsTRAIL is in clinical trials as a selective killer of tumor cells either alone or in combination with chemotherapeutic drugs.

Five distinct TRAIL receptors have been identified: DR4 (TRAIL-R1) (Pan, O'Rourke et al. 1997), DR5 (TRAIL-R2) (Walczak, Degli-Esposti et al. 1997; Wu, Burns et al. 1997), DcR1 (TRAIL-R3) (Sheridan, Marsters et al. 1997), DcR2 (TRAIL-R4) (Pan, Ni et al. 1997) and *O*steoprotegerin (OPG) (Emery, McDonnell et al. 1998). TRAIL receptors have been localized to human chromosome 8p21-22 (Walczak, Degli-Esposti et al. 1997; Ashkenazi and Dixit 1998). All these receptors have high sequence homology in their extracellular domains (Lee, Lee et al. 2005). They can be classified into two groups,

proapoptotic receptors (DR4 and DR5) and antiapoptotic receptors (DcR1, DcR2 and OPG) (LeBlanc and Ashkenazi 2003). DR4/5 is a type I membrane protein of 468/440 AA, containing two extracellular cysteine-rich pseudo-repeats and an intracellular death domain (DD) similar to the one of TNF-R1, DR3, Fas and CAR1 within the cytoplasmatic domain. DR5 has a homology of 58% with DR4. Both DR4 and DR5 contain a conserved DD motif and signal apoptosis (Pan, O'Rourke et al. 1997; Walczak, Degli-Esposti et al. 1997; Wu, Burns et al. 1997). The other three receptors appear to act as 'decoys' for their ability to inhibit TRAIL-induced apoptosis when overexpressed. DcR2 has a truncated, nonfunctional cytoplasmic DD while DcR1 lacks a cytosolic region and is anchored to the plasma membrane through a glycophospholipid moiety (Pan, Ni et al. 1997; Sheridan, Marsters et al. 1997). OPG binds TRAIL but has lower affinity at physiological temperature (Emery, McDonnell et al. 1998). A recent study suggests that cancer-derived OPG may be an important survival factor in hormone-resistant prostate cancer cells and there is a negative correlation between the levels of OPG and the capacity of TRAIL to induce apoptosis in prostate cancer cells that endogenously produce a high level of OPG (Holen, Croucher et al. 2002). In mice, there is only one death-inducing receptor for TRAIL, a protein homologous to human DR5 (mTRAIL-R, mDR5) (Wu, Burns et al. 1999).

As shown in Figure 1.1, binding of TRAIL to its proapoptotic receptors DR4 and DR5 triggers trimerisation of the receptors and formation of the *d*eath-*i*nducing *s*ignaling *c*omplex (DISC) by recruiting the adaptor molecule *Fas-associated death domain* (FADD), initiator caspase-8 and 10, followed by activation of caspase-8 and 10 and in turn caspase-3 which directly triggers a caspase cascade leading to apoptosis. Caspase-8

and 10 activation at the DISC can be inhibited by the cellular FLICE-inhibitory protein (cFLIP, FLICE is the old name of caspase 8) short form (cFLIP_s) and high levels of long form (cFLIP_L) which has two death effector domains (DEDs) similar to caspase-8 and 10 but be promoted by low levels of cFLIP_L (Chang, Xing et al. 2002; Yang 2002). Based on the requirement of the intrinsic pathway for apoptosis mediated by death receptors, cells can be divided into two types (Ozoren and El-Deiry 2002; Barnhart, Alappat et al. 2003). In type I cells, activation of caspase-8 in the DISC is sufficient to induce apoptosis. Peripheral T cells and thymocytes, human non small cell lung cancer cell line H460, human colon cancer cell line SW480 and HT29, human B lymphoblastoid cell line SKW6.4, human T lymphoma cell line HuT78, human Burkitt's lymphoma cell line BJAB and Raji, human hepatocellular carcinoma cell line HepG2, and human T lymphocyte H9 are type I cells (Scaffidi, Fulda et al. 1998; Scaffidi, Schmitz et al. 1999; Ozoren and El-Deiry 2002; Barnhart, Alappat et al. 2003). However, in type II cells, only a small amount of FADD and caspase-8 are recruited to the DISC, so insufficient activation of caspase-8 requires further amplification of the signal for apoptosis activation. This occurs through caspase 8-dependent cleavage of the BH3-only protein Bid, which then engages the intrinsic pathway to produce greater effector caspase activity (Scaffidi, Fulda et al. 1998). Hepatocytes, human esophageal cells, Lymphoblast CEM, T leukaemia cell line Jurkat, pre-B-cell line Boe^R, human epithelial adenocarcinoma cells HeLa, human colon cancer cell line HCT116 are type II cells (Scaffidi, Fulda et al. 1998; Scaffidi, Schmitz et al. 1999; Ozoren and El-Deiry 2002; Barnhart, Alappat et al. 2003). TRAIL-induced apoptosis also involves the intrinsic pathway in type II cells. Release of Smac/Diablo from mitochondria to block the caspase-3 inhibitory effect of the X chromosome-linked *i*nhibitor of *a*poptosis *p*roteins (XIAP) is required for type II cells to undergo apoptosis in response to TRAIL. Blockade of the intrinsic pathway by overexpression of antiapoptotic Bcl-2 proteins such as Bcl-2 and Bcl-X_L or inactivation of Bax may change TRAIL sensitivity in type II cells (Ozoren and El-Deiry 2002). A recent long-term clonogenicity study suggests that the mitochondrial pathway is essential for apoptotic execution of Type I tumor cells by death receptors: the release of Smac/DIABLO and the inhibition of XIAP activity proved to be crucial for full effector caspase activity and clonogenic execution in Type I tumor cells (Maas, Verbrugge et al. 2010).

1.3 TRAIL proapoptotic receptor-targeted therapy

Conventional chemotherapy kills rapidly dividing cells including both tumor and normal cells causing side effects like hair loss, nausea, diarrhea and anemia. But targeted therapies aim at tumor growth or survival associated proteins that ideally are not found in healthy cells. Hence targeted therapies can improve treatment response but with fewer side effects, which accelerates the pace toward personalized therapy. The apoptosis pathway triggered by the TRAIL proapoptotic receptors DR4/5 is a potential target for anticancer therapeutic intervention. Promoting apoptosis specifically in tumor cells via activation of DR4/5 has generated some of the most advanced and promising cancer therapies in development. As summarized in Table 1.1, current *proa*poptotic receptors DR4 and DR5 with *r*ecombinant *h*uman TRAIL (rhTRAIL) and several single receptor-targeting agonistic monoclonal antibodies such as HGS-ETR1 (Mapatumumab, TRM-1) and HGS-ETR2 (Lexatumumab, TRM-2) developed by Human Genome Sciences

(Ashkenazi and Herbst 2008). There are 25 ongoing clinical trials targeting TRAIL proapoptotic receptors (Russo, Mupo et al. 2010). Combining knowledge on the TRAIL signaling pathway with PARA clinical response data and tumor characteristics may give rise to true tumor-tailored therapy.

Soluble rhTRAIL is unique in that it activates both DR4 and DR5. Preclinical data indicate that rhTRAIL can induce apoptosis in a broad range of human cancer cell lines while sparing most normal cell types. RhTRAIL exhibits single-agent antitumor activity and/or cooperation with certain conventional and targeted therapies in vitro and in various in vivo tumor xenograft models. Safety studies in nonhuman primates have shown rhTRAIL to be well tolerated. After a single intravenous (IV) bolus administration, rhTRAIL exhibited a serum half-life $(t_{1/2})$ of approximately 3 to 5 minutes in rodents and approximately 23 to 31 minutes in primates. It is primarily eliminated through the kidneys (Duiker, Mom et al. 2006). Despite a very short $t_{1/2}$ of rhTRAIL in mouse and the estimated steady-state volumes of distribution indicating that rhTRAIL may not distribute greatly outside of the vascular space prior to its elimination from the system, rhTRAIL has demonstrated significant antitumor activity in mice containing human xenografts (Kelley, Harris et al. 2001). Rapid clearance was observed after a 1-hour IV infusion of rhTRAIL at 10~100 mg/kg in cynomolgus monkeys, with a serum $t_{1/2}$ of 28 to 30 minutes after infusion of the highest dose. The pharmacokinetics (PK) studies suggest a dosedependent profile that does not lead to accumulation over time. The relatively short serum $t_{1/2}$ may require frequent administration. Further optimization of dose and regimen has been under investigation (Ashkenazi, Holland et al. 2008). In chimpanzees, clearance was linear over the dose range $1 \sim 50 \text{ mg/kg}$ and the area under the curve (AUC) of serum

drug concentration versus time and the maximal serum concentration (C_{max}) increased proportionally with dose (Kelley, Harris et al. 2001). A preclinical study demonstrated that cisplatin can sensitize resistant primary human hepatocytes to TRAIL (Ganten, Koschny et al. 2006). Moreover early clinical trial data suggest that rhTRAIL is generally safe and provide preliminary evidence for potential antitumor activity. A current clinical experimental treatment schedule is 1-hour rhTRAIL infusion for 5 days every 3 weeks. A phase Ia safety and PK study was conducted in patients with various advanced solid tumors or Non-Hodgkin lymphoma (NHL). RhTRAIL proved to be safe and well tolerated at dose 0.5~15 mg/kg in 58 patients with no drug-related dose-limiting toxicities (DLT) observed. Single-agent rhTRAIL up to 8 cycles yielded stable disease (SD) in 21 of 37 heavily pretreated patients who were assessable for response, and a validated partial response (PR) in one patient. Among reported 46 patients, rhTRAIL exhibited a linear PK profile consistent with preclinical studies. Hepatic metastases with or without mild liver dysfunction did not appear to influence the PK of TRAIL (Herbst, Mendelson et al. 2006; Ling, Herbst et al. 2006). The first reported single agent phase I, open-label, doseescalation study treated patients with advanced cancer with rhApo2L/TRAIL doses ranging from 0.5 to 30 mg/kg/d with parallel dose escalation, given daily for 5 days every 3 weeks. RhApo2L/TRAIL was safe and well tolerated. Overall 33 (46%) of 71 patients had stable disease or better at the end of cycle 2. Eight patients had stable disease for > 4months but < 6 months. Two patients (3%) with chondrosarcoma had confirmed partial responses at > 6 months (Herbst, Eckhardt et al. 2010). The first reported phase Ib study treataed 24 previously untreated, nonsquamous, stage IIIb (with pleural effusion)/IV or recurrent non-small-cell lung cancer (NSCLC) with rhApo2L/TRAIL at 4 or 8 mg/kg/d for 5 consecutive days or 15 or 20 mg/kg/d for 2 consecutive days every 3 weeks in combination with paclitaxel, carboplatin, and bevacizumab. The drug combination was well tolerated and demonstrated antitumor activity - one confirmed complete response and 13 confirmed partial responses (Soria, Smit et al. 2010). Phase II clinical trials evaluating the safety and efficacy of rhTRAIL in combination with established anticancer therapies are ongoing.

HGS-ETR1 and HGS-ETR2 are fully humanized agonistic monoclonal antibodies to DR4 and DR5. The advantages of the agonistic antibodies compared with TRAIL include a longer plasma $t_{1/2}$ and the lack of binding to decoy receptors (Buchsbaum, Forero-Torres et al. 2007). Pretreatment with cisplatin followed by HGS-ETR1 or HGS-ETR2 synergistically inhibited the cell growth and enhanced apoptotic death (Belyanskaya, Marti et al. 2007).

HGS-ETR1 reduced the viability of multiple types of tumor cells *in vitro*, and induced activation of caspase 8, Bid, caspase 9, caspase 3, and cleavage of the *p*oly (*A*DP-*r*ibose) *p*olymerase (PARP), indicating activation of DR4 alone was sufficient to induce both extrinsic and intrinsic apoptotic pathways. HGS-ETR1 enhanced the cytotoxicity of chemotherapeutic agents even in tumor cell lines that were not sensitive to HGS-ETR1 alone. In xenograft models, HGS-ETR1 induced rapid tumor regression or repression of tumor growth in a broad range of tumors. Combination of HGS-ETR1 with chemotherapeutic agents resulted in an enhanced antitumor efficacy compared to either agent alone (Pukac, Kanakaraj et al. 2005). Sequential treatment with paclitaxel followed by HGS-ETR1 or HGS-ETR2 after 48 h resulted in markedly enhanced antitumor activity against Colo205 mouse xenografts (Gong, Yang et al. 2006). Preclinical studies suggest

that HGS-ETR1 is a specific and potent antitumor agent with favorable PK characteristics and the potential to provide therapeutic benefit for a broad range of human malignancies (Pukac, Kanakaraj et al. 2005). A phase I safety, PK, and preliminary evidence of antitumor activity study in patients with advanced solid malignancies showed that HGS-ETR1 can be administered safely and feasibly at 10 mg/kg IV every 14 days, with $t_{1/2}$ of 18.8 days and mild adverse events. In a total of 49 patients, 19 patients had stable disease (SD) with two responses in patients lasting 9 months. Further disease-directed studies of HGS-ETR1 alone and in combination with chemotherapy in a broad array of tumors are warranted (Tolcher, Mita et al. 2007). A phase I open-label, dose-escalation study assessed the tolerability and toxicity profile in 41 patients with advanced solid tumors showed that HGS-ETR1 was well tolerated with escalation levels from 0.01 to 20.0 mg/kg. The maximum tolerated dose was not reached. Linear pharmacokinetics was observed for doses up to 0.3 mg/kg and for the 20 mg/kg level, whereas exposure at 3 and 10 mg/kg increased less than proportionally. 12 patients had SD for 1.9 to 29.4 months (Hotte, Hirte et al. 2008). A phase II multi-center study in 32 patients with advanced heavily pretreated NSCLC showed HGS-ETR1 was safe and well tolerated at 10mg/kg IV every 21 days. 9 patients (29%) had SD (Greco, Bonomi et al. 2008). Phase II and Phase Ib combination studies of HGS-ETR1 to evaluate its potential for the treatment of specific cancers have been initiated.

HGS-ETR2 has shown broad and significant activity across a broad range of cell lines *in vitro* and *in vivo* as a single agent and in combination with chemotherapy. A phase I dose-escalation safety, tolerability and PK study in patients with advanced solid malignancies showed HGS-ETR2 can be safely administered every 14 days at doses up to

10.0 mg/kg. In 31 patients, one patient experienced a dose-limiting toxicity (DLT) (grade 3 hyperamylasemia) at the 10 mg/kg dose level, 10 patients (32.3%) had SD. HGS-ETR2 PK is linear up to 10 mg/kg. At the 10 mg/kg dose, the $t_{1/2}$ was found to be 11 days. The large steady-state volume of distribution indicates that HGS-ETR2 distributes outside the plasma compartment (Sarantopoulos, Wakelee et al. 2005; Manzo, Nebbioso et al. 2009). A phase I open label study in patients with advanced solid malignancies showed that the PK was linear over the dose range of $0.1 \sim 20 \text{ mg/kg}$, DLT (asymptomatic elevations of serum amylase, transaminases, and bilirubin) dose is 20 mg/kg, the maximum tolerated dose is 10 mg/kg, $t_{1/2}$ at the 10 mg/kg IV every 21 days is 16.4 days. In 37 patients, 12 patients had durable SD. HGS-ETR2 was safe and well tolerated at doses ≤10 mg/kg every 21 days (Plummer, Attard et al. 2007). A phase Ib HGS-ERT2 in combination with chemotherapy study in 41 patients with a wide range of solid malignancies demonstrated that HGS-ETR2 at 5 mg/kg or 10 mg/kg was generally safe and well tolerated in combination with full-dose different standard chemotherapy regimens. Objective responses were reported for two patients and 22 patients had SD. The PK of HGS-ETR2 was not influenced by the chemotherapeutic agents and vice versa. Further studies of HGS-ETR2 in combination with chemotherapy are warranted (Sikic, Wakelee et al. 2007).

1.4 Sorafenib

The well-tolerated targeted agents provide new options for the combination treatment of patients with advanced solid tumors. The RAF/MEK/ERK pathway and upregulation of VEGFR both play important roles in the growth and maintenance of solid tumors. Thus blocking these signaling pathways will be another promising target for cancer therapy.

The novel bi-aryl urea Sorafenib (Nexavar®; Bayer Pharmaceuticals Corporation, Onyx Pharmaceuticals) is an oral multi-kinase inhibitor with novel dual action Raf serine/threonine kinases (Raf-1, wild-type B-Raf, and *b-raf* V600E) and receptor tyrosine kinases (the vascular endothelial growth factor receptor (VEGFR)-1/-2/-3, plateletderived growth factor receptor- β (PDGFR- β) and Flt-3, c-Kit, and p38 tyrosine kinases) inhibition that targets tumor cell proliferation and tumor angiogenesis (Wilhelm, Carter et al. 2004). The mechanism of action of Sorafenib is competitive inhibition of ATPbinding to the catalytic domains of the respective kinases (Wilhelm and Chien 2002; Huether, Hopfner et al. 2007). Targeting multiple pathways makes Sorafenib more favorable since cancer may be altering multiple targets. Sorafenib is generally well tolerated and synergizes with many standard chemotherapy agents and cytotoxic agents, and has shown promise in a variety of malignancies including gastrointestinal malignancies, renal cell carcinoma, hepatocellular carcinoma and lung cancer (Vincent, Zhang et al. 2002; Heim, Sharifi et al. 2003). Sorafenib has demonstrated preclinical and clinical anticancer activity as a monotherapy and in combination with other anticancer agents. Proven effective as a single-agent therapy in renal cell carcinoma, Sorafenib was approved in the U.S. in December 2005 (Dal Lago, D'Hondt et al. 2008). Sorafenib is the first approved systemic drug therapy for unresectable *h*epatocellular carcinoma (HCC), as well as being the only medication therapy proven to significantly improve overall survival in HCC patients (http://www.cancer.gov/cancertopics/druginfo/fda-Sorafenibtosylate). The safety, PK, and anti-tumor activity of Sorafenib combined with other targeted agents or cytotoxics from Phase I/II trials were reviewed by Takimoto (Takimoto and Awada 2008). In brief the recommended dose of Sorafenib for combination is 400

mg twice daily. Sorafenib was well tolerated with mild adverse events and had little effect on the PK of coadministered agents and *vice versa*. Overall disease control rates (PR plus SD) ranged from 33 to 92% (Takimoto and Awada 2008). The antiproliferative and antiangiogenic mechanisms of Sorafenib in combination with other anticancer agents with complementary or contrasting mechanisms such as PARAs are potentially valuable new treatment regimens for patients with advanced solid tumors. The combination therapy of Sorafenib and TRAIL appears to be rational, much less toxic, and dramatically effective on TRAIL-resistant tumors in preclinical trials. The synergistic effects of combining Sorafenib with TRAIL include sensitization to TRAIL induced apoptosis, inhibiting tumor growth, reducing levels of TRAIL induced antiapoptotic factors such as Mcl-1 Bcl-X_L and cIAP2, and increasing proapoptotic factors Bak, Bid and Bax activation (Meng, Lee et al. 2007; Ricci, Kim et al. 2007; Rosato, Almenara et al. 2007). Currently there is an ongoing clinical trial "A Study of Mapatumumab in Combination With Sorafenib in Subjects With Advanced *H*epatocellular *Carcinoma* (HCC)".

1.5 Molecular imaging in cancer therapy response prediction

Predicting tumor responsiveness to treatment at an early phase would be very useful to identify responders and avoid prolonged use of ineffective therapies. A variety of routine anatomical imaging modalities are used to evaluate treatment efficacy. However changes in tumor physiology, proliferation and metabolism often precede these volumetric changes. Therefore imaging these early changes will help to assess or even predict treatment response (Verheij 2008). Molecular imaging can be used to find out early whether an agent is having a measurable effect on a biological endpoint. To predict therapy response using molecular imaging, the most straightforward approach is direct

visualization of target and ligand-target binding using labeled ligands. Nuclear imaging and in particular positron emission tomography (PET) have been used to study receptor occupancy. In studies related to PARAs, radiolabeled receptor antibodies were used to assess receptor expression level and receptor occupancy with gamma camera and PET respectively (Gong, Yang et al. 2006; Hwang, Rossin et al. 2008). Optical-based molecular imaging is an emerging field with the advantages of cost-effective, rapid, easy to use, and ready for *in vivo* studies. Optical molecular imaging plays an important role in oncologic drug development in both preclinical and clinical settings. Light in the near infrared (NIR) range (650–900 nm) can traverse tissue very efficiently as the absorption by water and endogenous chromophores such as hemoglobin, melanin, and lipids is relatively low in this spectrum. Tissue autofluorescence is minimal at 800 nm. So an optimal wavelength for imaging is centered at 800 nm. Indocyanine green (ICG) has been used as the only FDA approved NIR dye for clinical retinal angiography. But ICG does not have a reactive group for conjugation to a targeting moiety for molecular imaging. ICG is hydrophobic and is bound nonspecifically to serum proteins. The excitation and emission of IRDye 800CW (LI-COR Biosciences) is in the spectral region where tissue absorption, autofluorescence, and scattering are minimal yielding the highest signal to background ratios. By contrast, IRDye 800CW reactive dyes can be easily conjugated to a variety of targeting agents. IRDye 800CW is highly water soluble and shows very low nonspecific binding to cellular components and yields very high signal. In animal studies, it has been shown to clear through the kidneys and is approximately twenty times brighter than ICG. Recently a single-dose intravenous toxicity study of IRDye 800CW was performed. Intravenous dose levels of 1, 5, and 20 mg/kg or intradermal 20 mg/kg

produced no pathological evidence of toxicity (Marshall, Draney et al. 2010). Targeting agents such as small polypeptide growth factors, monoclonal antibodies, peptide analogues of extracellular ligands endostatin and somatostatin, labeled with Cy5.5, Alexa Fluor® 680, or IRDye 800CW, have been used successfully for optical noninvasive detection of tumor xenografts in mice. EGF labeled with Cy5.5 was used to quantify therapeutic efficacy of anti-EGF receptor antibodies for treating mammary carcinoma in mice (Kovar, Johnson et al. 2006; Kovar, Volcheck et al. 2007).

The presence of DR4 and DR5 on the tumor cell surface is a necessary but not always sufficient prerequisite for antitumor activity induced by PARAs. Efficacy biomarkers targeting general tumor properties such as proliferation, angiogenesis and apoptosis of tumors are therefore exploited. Increased cellular proliferation is a hallmark of the cancer. Thymidine kinase activity increases about 10-fold as cells enter the DNA synthetic phase (Choy, Choyke et al. 2003). Cell proliferation imaging assesses the uptake and metabolism of nucleosides used in nucleic acid synthesis. Monophosphorylation of the thymidine nucleoside analogue $3'-[^{18}F]$ fluoro-3'-deoxy-L-thymidine (^{18}F -FLT) by intracellular thymidine kinase-1 (TK-1) results in FLT trapping in proliferating cells. FLT images reflect TK-1 activity and the percentage of cells in S phase (Rasey, Grierson et al. 2002). PET is emission computed tomography that is used to study the distribution of radiolabeled tracers within the body. PET radiotracers emit positrons that, after encountering electrons, lead to emission of 2 gamma rays directed at 180 degrees from each other. Sites of tracer accumulation in the body are thus determined by coincidence detection of these paired gamma rays. FLT-PET is thus used to measure cellular TK-1 activity and thereby infer the rate of cellular proliferation. Treatment response was

predicted by tumor FLT uptake changes in as early as 24 hours after the initiation of treatment (Barthel, Cleij et al. 2003; Sugiyama, Sakahara et al. 2004; Waldherr, Mellinghoff et al. 2005; Apisarnthanarax, Alauddin et al. 2006; Chao 2006; Yang, Ryu et al. 2006; Buck, Kratochwil et al. 2007; Chen, Delaloye et al. 2007; Herrmann, Wieder et al. 2007; Molthoff, Klabbers et al. 2007; Salskov, Tammisetti et al. 2007; Solit, Santos et al. 2007; Yamamoto, Nishiyama et al. 2007). Compared to current diagnosis and staging using 2-[¹⁸F]/luoro-2-deoxy-D-glucose (¹⁸F-FDG)-PET, FLT-PET has lower sensitivity. But the specificity of FLT-PET for assessment of cellular proliferative activity makes it an ideal tracer for monitoring tumor treatment response. FLT-PET is correlated much better with the proliferation marker Ki67 than FDG-PET (Buck, Halter et al. 2003; Apisarnthanarax, Alauddin et al. 2006). FDG-PET cannot distinguish a pathologic complete response from residual tumor of up to 50% in correlation of postchemoradiation FDG standardized uptake value (SUV) and percent residual tumor in patients with esophageal cancer (Swisher, Erasmus et al. 2004). SUV is defined as the tissue concentration of tracer as measured by a PET scanner divided by the activity injected divided by body weight (Zasadny and Wahl 1993). Moreover FDG-PET cannot differentiate cancer response to radiotherapy from the local normal tissue inflammation caused by radiotherapy. Effective cancer treatment can occur by inducing cytostasis rather than cytotoxicity because cytostasis is often poorly tolerated in tumor cells and should lead to cell death if sustained (Rixe and Fojo 2007). Cytostatic agents stop cancer cells from proliferating. Clinically, cytostasis would result in SD. Many of the compounds now being developed are not going to lead to tumor shrinkage; the best

response may be SD (Herbst 2004). As an imaging marker of cellular proliferation, FLT-PET outshines other imaging modalities in this instance.

The early apoptotic response observed prior to tumor volume changes correlates well with subsequent outcome (Meyn, Stephens et al. 1995; Ellis, Smith et al. 1997; Symmans, Volm et al. 2000; Bremer, Ntziachristos et al. 2003). Phosphatidylserine (PS) externalization is one of the most widely used markers for apoptotic cell death in mammals. Multimodality imaging of the PS-binding protein Annexin V including scintigraphy, optical imaging, multispectral imaging, single-photon emission computed tomography (SPECT) and MRI has been used to assess apoptosis right after the initiation of proapoptotic therapy (Belhocine, Steinmetz et al. 2002; Schellenberger, Bogdanov et al. 2002; Petrovsky, Schellenberger et al. 2003; Mandl, Mari et al. 2004; Ntziachristos, Schellenberger et al. 2004; Dicker, Kim et al. 2005; Henery, George et al. 2008). In vivo imaging of apoptosis offers another noninvasive approach to monitor therapeutic response independent of changes in glucose metabolism or cell proliferation. Imaging malignant angiogenesis is also employed to evaluate treatment response, i.e., effective blockage of tumor vascular neoangiogenesis (Runnels, Zamiri et al. 2006; Backer, Levashova et al. 2007; Saxena, Gonzalez-Gomez et al. 2007; Yang, Jiang et al. 2007).


Figure 1.1 TRAIL signaling pathway (Reprinted from (Falschlehner, Emmerich et al. 2007) Copyright © 2007, Elsevier, Used by Permission)

Agent	Mechanism	Synonyms
Apo2L/TRAIL	Targeting DR4 and DR5	Dulanermin, AMG 951
TRAIL-R1 mAb	Targeting DR4	HGS-ETR1, Mapatumumab, TRM-1
TRAIL-R2 mAb	Targeting DR5	HGS-ETR2, Lexatumumab, TRM-2,
		AMG 655, CS-1008, LBY135,
		PRO95780, Drozitumab

Table 1.1 Proapoptotic receptor agonists

CHAPTER 2

Multispectral Fluorescence Imaging

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2.1 Abstract: *M*ultispectral *f*luorescence *i*maging (MSFI) is a rapidly growing field with broad applications in both preclinical and clinical settings. Application of this novel technology in small animal imaging and microscopy produces enhanced sensitivity, reliable quantification, and resolves multiple simultaneous signals. MSFI flow cytometry can quantify multiple fluorescent parameters with morphological or sub-cellular spatial details on millions of cells. MSFI has the potential to improve the accuracy of disease detection/differentiation and intrasurgical metastatic diagnosis, guide neurosurgeries, and monitor treatment response.

Key Words: multispectral fluorescence imaging, multiplexing, spectral unmixing, small animal imaging, microscopy, flow cytometry

*M*ultispectral *i*maging (MSI) is the synergistic combination of imaging and spectroscopy. Color is the appearance of a light most affected by wavelength (λ) and the observers' visual system. Objects with similar colors are not necessarily the same. The simplest example is the spectrally pure yellow and the mixture of red and green, which have completely different spectral content but precisely the same *r*ed-green-*b*lue (RGB) coordinates (Bearman and Levenson 2003). Spectroscopy is the technique of breaking light down into its composite colors to identify the object's composition. Distinguished from conventional RGB full-color imaging, MSI can obtain a high resolution optical spectrum for each image pixel, resulting in a series of images of the same field of view that are acquired at different wavelengths. These can be stacked in three dimensional datasets or a "cube" (x, y, λ). After proper calibration, quantitative images of individual analytes can be generated.

There are excellent reviews of MSI instrumentation and data analysis (Hiraoka, Shimi et al. 2002; Bearman and Levenson 2003). Briefly, a camera is used to acquire the spatial information and the spectral information is gained by scanning a dispersive element to record spectra for each image. Electronic tunable filters such as acousto-optic tunable filter (AOTF) and liquid crystal tunable filters (LCTFs) are preferable to mechanically scanning dispersive devices (filter wheels, monochromators) because they are quiet, fast, compact and stable, and demonstrate increased spectral selectivity, spectral purity and flexibility. The disadvantages of electronic tunable filters include low light throughput, photobleaching and inappropriate capture if significant sample or camera movement occurs during the acquisition, or if high temporal resolution is needed to capture certain events (e.g., calcium signaling transients) (Levenson and Mansfield 2006). The typical method for data analysis of MSI is a least-squares fitting linear unmixing approach with additional constraints (such as non-negativity), resulting component images and a final composite image. This approach is limited to spectral analysis only whereas combining the rich spatial information in the images with the spectral data is a more powerful and evolving field (Bearman and Levenson 2003). MSFI coupled with microscopy and flow

cytometry are commercially available now (Zuba-Surma, Kucia et al. 2007; Mansfield, Hoyt et al. 2008).

MSI technologies have been widely used in the fields of astronomy, geology, agriculture, industry and forensics (Hiraoka, Shimi et al. 2002; Bearman and Levenson 2003). With the vast development of filters, detectors, data analysis techniques, interdisciplinary approaches and fluorescent dyes, MSFI is a rapidly growing field with applications in cell biology, preclinical drug development and clinical pathology. Combining *Multispectral fluorescence imaging* (MSFI) with small animal imaging and microscopy produces enhanced sensitivity, reliable quantitation, and resolved multiple simultaneous signals (Mansfield, Gossage et al. 2005; Levenson and Mansfield 2006). MSFI is particularly useful for analyzing objects that have multiple fluorescent labels that may have similar RGB color or that may be localized in the same or spatially overlapping compartments; this modality is also effective for evaluating objects that have strong whole-animal autofluorescence (Figure 2.1) (Levenson, Lynch et al. 2008; Mansfield, Hoyt et al. 2008; van der Loos 2008). Here we review a selection of preclinical and clinical studies describing the use of MSFI applications.

2.2 Preclinical application of multispectral fluorescence imaging

2.2.1 To assess tumor vascularization and monitor the antiangiogenic therapeutic response

Previous studies imaged tumor angiogenesis with fluorescent proteins (Hoffman 2004; Hoffman 2009). MSFI provides a powerful, non-invasive means by which to assess tumor vascularity and to monitor the response to anti-angiogenic therapy. For example, in mice treated with sunitinib, a near infrared dye labeled monoclonal antibody-based probe targeting VEGFR2 was imaged in vivo using a LCTF-based Maestro MSFI system (CRI Inc., Woburn, MA). Tumor fluorescence intensity was quantified and normalized after autofluorescence removal. This study demonstrates that MSFI provides rapid, noninvasive and longitudinal assessment of the efficacy of novel angiogenesis-directed therapies in preclinical models of cancer (Virostko, Xie et al. 2009). To overcome constraints associated with exogenously administered vascular imaging probes such as high cost, toxicity, inconsistent delivery, differential bioavailability among animals and high tumor accumulation resulting from vessel leakiness, a novel method to noninvasively image tumor vascularization in fluorescent tumors without exogenous imaging probes was introduced. LCTF-based Maestro and Nuance systems (CRI Inc., Woburn, MA) were used for *in vivo* MSFI to study the biology of tumor angiogenesis and to monitor the effects of antiangiogenic therapies with bevacizumab. In a mouse model bearing tumors expressing red fluorescent protein (RFP), the spectral fluorescence signatures of vascular and avascular components allow for the tumor vasculature to be imaged and quantified without contrast agents (Figure 2.2). This technique provides realtime imaging of tumor vascularization and monitoring the antiangiogenic therapeutic response in mice without the concerns specific to an exogenously administered probe (Mayes, Dicker et al. 2008).

2.2.2 To generate region-selective brain maps of vascularized brain parenchyma

A FITC-derivatized tomato lectin - based molecular imaging probe was utilized to generate region-selective brain maps of vascularized brain parenchyma *ex vivo* in rats and rabbits with the Maestro system. Images were unmixed to get rid of brain tissue autofluorescence and resolve arterial blood and specific imaging probe fluorescence

signal. After further refinement of imaging probes and metrics, this novel preoperative endovascular brain mapping approach may facilitate neurosurgical guidance and therefore improve clinical outcomes after neurosurgical resections (Manning, Shay et al. 2009).

2.2.3 Automatable analysis of multiplexed immunolabeled samples

More than six markers can be quickly differentiated from each other and from autofluorescence using MSFI thereby allowing for the localization of multiple probes and avoid the need for serial sections. For example, Mansfield et al. performed six-marker analyses in one sample with MSFI. Quantitative and marker-specific image data were presented for integrative system biological studies. The drawbacks of this approach are too much work to prepare a series of controls of all of the combinations of markers and the method is less optimal for membrane or cytoplasmic marker analyses (Mansfield, Hoyt et al. 2008).

2.2.4 To differentiate tissue type and provide cellular level diagnostic information

The slit-scan confocal microendoscope uses a catheter based on a fiber optic imaging bundle. This multispectral system uses a prism as a dispersive element to collect a full multispectral image with spectral resolution of 2.9 nm at 500 nm and 8.4 nm at 750 nm. Human ovary and esophagus and mouse peritoneal wall and liver stained with acridine orange and rat intestine tagged with DiA (4-*Di*-16-*A*SP, 4-(4-(dihexadecylamino)styryl)-N-methylpyridinium iodine, Invitrogen) were imaged *ex vivo*. This method of MSFI can distinguish tissue types and acquire high quality images. It is minimally invasive and has potential to provide cellular level *in vivo* diagnostic information as a clinical diagnostic instrument (Makhlouf, Gmitro et al. 2008).

QDs are novel nanocrystal fluorophores with constant broad excitation spectra, sharp and symmetrical tunable emission spectra, improved brightness, superior photostability and simultaneous excitation of multiple fluorescence colors. QDs have been successfully applied in various imaging applications for *in vitro* diagnostics and *in vivo* imaging because of these unique optical properties. MSFI-QDs can efficiently remove background and precisely delineate weak spectral signatures to present ultrasensitive and multiplexed imaging of molecular targets in vivo (Gao, Cui et al. 2004; Su, Zhang et al. 2008). Full reviews on MSFI-QDs in multiplexed immunohistochemistry and *in situ* hybridization (ISH) are available (Tholouli, Sweeney et al. 2008; Xing and Rao 2008). Another use of MSFI-QDs is as a feasible substitute for microarray analysis. In this instance, multiplexed QD-labeled oligonucleotide probes can be used for ISH in human biopsies. This technique was tested in a study in which single and multiplexed QD-ISH was performed in samples with acute leukemia and follicular lymphoma. Spectral unmixing enables the separation of spatially colocalized signals. MSFI-QDs allow quantitative characterization of multiple gene expression thereby making it useful for the analysis of processed human clinical tissues (Tholouli, Hoyland et al. 2006). MSFI-QDs were also tested using streptavidin-conjugated QDs to detect up to seven signals in tonsil and lymphoid tissue. Slides were analyzed using confocal laser scanning microscopy. Five streptavidinconjugated QDs were used on the same tissue section and could be analyzed simultaneously on routinely processed sections (Figure 2.3). This multiplexing method has the potential to uncover the clinically relevant multidimensional cellular interactions that underlie diseases (Fountaine, Wincovitch et al. 2006).

2.3 Clinical application of multispectral fluorescence imaging

2.3.1 Combining MSFI with two-photon excitation (TPE) to detect cutaneous tumors

TPE fluorescence microscopy offers high spatial resolution, deep penetration and low photodamage. To detect cutaneous tumors, combined TPE and MSFI system (MTPE) employed to identify different intrinsically fluorescent molecules (NADH was (*n*icotinamide adenine dinucleotide), tryptophan, keratins, melanin. elastin. cholecalciferol and others). This technique provides functional information correlated with tissue structure in physiological and pathological states. MTPE is effective in differentiating healthy skin from basal cell carcinoma (De Giorgi, Massi et al. 2009). As more samples are analyzed, it should be possible to differentiate between different types of malignancies as well.

2.3.2 Application of multispectral imaging flow cytometry

MSI flow cytometry can quantify multiple fluorescent parameters with morphological or sub-cellular spatial details on millions of cells (For reviews see references (Zuba-Surma, Kucia et al. 2007; McGrath, Bushnell et al. 2008)). Nuclear translocation of NF κ B initiates the transcription of host defense related critical genes. To quantitatively measure the translocation of signaling molecules from the cytoplasm to the nucleus in cells, cells were stained with FITC-conjugated anti-NF κ B and 7-AAD (DNA stain) and imaged by flow cytometry. A similarity score that quantifies the correlation of the spatial distribution of the stained, translocating signaling molecule with nuclear staining was generated for each cell using Pearson's correlation coefficients to assess the nuclear translocation. This approach represents an advance over conventional microscopy or biochemical fractionation and western blotting for high throughput single cell or subpopulation analysis (George, Fanning et al. 2006; George, Morrissey et al. 2009). MSI flow cytometry can not only distinguish live cells from cells in the early or late phases of apoptosis or from necrotic cells, (George, Basiji et al. 2004) but also accurately quantitate apoptosis based on morphological characterization and allow more complete and robust analysis of apoptosis than conventional microscopy (Henery, George et al. 2008). Covalent deposition of C3 fragments (C3b/iC3b) on cells is closely associated with cell-bound chimeric humanized anti-CD20 mAb Rituximab (RTX). To quantitatively analyze co-localization of C3 with RTX, Raji cells were stained with fluorescently-labeled RTX and mAbs specific for C3b/iC3b, and then imaged in flow and analyzed for co-localization of fluorescent probes. Analysis verified that the majority of deposited C3b/iC3b is co-localized with bound RTX (Beum, Lindorfer et al. 2006).

2.3.3 Autofluorescence (AF) analysis in MSFI

MSFI of oral tissue AF *in vivo* with a four channel multispectral digital microscope can improve detection of oral neoplasia. Decreased blue/green AF and increased red AF in lesions were observed in patients with histologically confirmed neoplasia (Roblyer, Richards-Kortum et al. 2008). MSFI of the ocular fundus AF may differentiate *a*ge related *m*acular *d*egeneration (AMD) and *d*iabetic *r*etinopathy (RD). AF in AMD patients was dominated by the red emission while RD green-shifted. Lipofuscin is the dominant fluorophore in the red channel. The green shift in RD may be related to increased advanced glycation end product concentrations (Hammer, Konigsdorffer et al. 2008). MSFI of sentinel lymph node AF during surgery can provide a rapid method to determine whether a tumor has metastasized to help determine follow up treatment. For example, to evaluate lymph nodes in colorectal and gastric tumors, AF microspectroscopy and MSI AF microscopy have been used to analyze the AF emission of metastatic lymph node sections. The AF pattern and the emission spectrum of metastatic lymph nodes can distinguish them from the normal ones (Pantalone, Andreoli et al. 2007). MSFI of AF has potential for detecting oral neoplasia, discriminating fundus autofluorescence fluorophores and intrasurgical diagnosis of metastatic lymph nodes.

2.3.4 Spatial-spectral analysis in MSFI

Using exclusive spectral information from a LCTF-based MSFI system with image analysis software (Environment for Visualizing Images, ENVI) can identify malignant urothelial cells in urine cytology specimens with a sensitivity and specificity of 82% and 81%, respectively or combined sensitivity and specificity of 73% (Jaganath, Angeletti et al. 2004). To increase the accuracy and specificity of the diagnosis and follow-up of bladder cancer, the spatial-spectral features of multispectral image stacks acquired with the same MSFI system were analyzed using *GEN*etic Imagery Exploitation (GENIE) package (Los Alamos National Laboratory). GENIE showed a combined sensitivity and specificity of 85 and 95% in samples from two separate institutes. Furthermore, GENIE showed efficiency superior to a cytopathologist with respect to predicting the follow-up results (Angeletti, Harvey et al. 2005). MSFI of fine-needle aspirations (FNAs) of follicular adenoma and parathyroid adenoma with the Nuance system and spatial-spectral analysis with CRI-MLS software can differentiate and classify morphologically similar lesions. The best spatial-spectral imaging solution has a sensitivity of 98.5%, a specificity of 96.1% and a positive predictive value of 98.6% (Mansoor, Zalles et al. 2008). Spatialspectral analysis is a promising tool for improving detection of bladder carcinoma when

morphology is not enough for a definitive diagnosis and differentiation of lesions with subtle cytomorphologic differences.

We have reviewed how MSFI can increase sensitivity and reliable quantification in preclinical and clinical applications. MSFI has been widely used in applications ranging from flow cytometry, microscopy, endoscopy, small animal imaging to human imaging. With the development of imaging devices, data analysis technologies and fluorescent dyes, MSFI has the potential to improve the accuracy of disease diagnosis and predict treatment response.



Figure 2.1 Five-color spectrally unmixed quantum dot detection of lymphatic system **anatomy.** (A) A schematic illustration of 5-color quantum dot lymphatic injection sites and draining destinations of spectral fluorescence imaging, with a graph of the emission spectra of each of the quantum dots used. Five primary draining lymph nodes were simultaneously visualized with different colors as shown: autofluorescence image of mouse (B); composite pseudo-colored detection of draining lymph nodes after spectral unmixing (C); image merge of the left and center panels (D). Reflectance (E) and spectrally unmixed and pseudo-colored image (F) of surgically dissected lymph nodes arranged in the same geometry as in the intact mouse. (This figure originally appeared in an article by Richard M. Levenson et al. in ILAR Journal 49(1). (Levenson, Lynch et al. 2008) It is reprinted with permission from the ILAR Journal, Institute for Laboratory Animal Research, The National Academies, Washington DC www.nationalacademies.org/ilar).



Figure 2.2 Noninvasive imaging of tumor vasculature using multispectral unmixing in fluorescent tumors. (A) The spectral fluorescent signatures of vascular components (emission wavelength maximum or peak emission $[Em_{max}]=620$ nm, red spectra) and avascular components ($Em_{max}=610$ nm, green spectra). (B) An image of a fluorescent tumor taken using the CRi Nuance camera attached to an AZ100 dissecting microscope from Nikon. (C) A high magnification image of the same tumor area as (B) showing the ability to detect capillaries (5–10 µm in diameter) within fluorescent tumors using multispectral unmixing. (D) Multispectral unmixing of a macroscopic whole body image of a mouse with fluorescent tumors on both rear flanks (left panel). Enlarged image of the left rear flank tumor (right panel). (E) Using the CRi software, a custom-threshold *r*egion *of i*nterest (ROI) is created in the vascular and avascular component images. A vascular index is then calculated by dividing the avascular component total area (mm²) by the vascular component total area (mm²). (Reprinted from (Mayes, Dicker et al. 2008) © 2008 BioTechniques, Used by Permission)



Figure 2.3 Quintuplet streptavidin-conjugated quantum dots multiplex and emission fingerprint of the multiplexed quantum dots (germinal center outlined with dotted line). (a) DAPI nuclear counter stain 450 nm (white). (b) CD 20 streptavidin-conjugated quantum dot 525 nm (red). (c) IgD streptavidin-conjugated quantum dot 565 nm (cyan). (d) MIB-1 streptavidin-conjugated quantum dot 605 nm (green). (e) CD 3 streptavidin-conjugated quantum dot 655 nm (yellow). (f) CD 68 streptavidin-conjugated quantum dot 705 nm (white). (g) Overlay of (b–f) including IgD (cyan) and CD20 (red) coexpression (magenta, see arrows and inset). (h) Emission profile generated from Lambda scan from 395 to 715 nm. Dotted lines are the single emission profile from DAPI alone or each of the streptavidin-conjugated quantum dots alone. Solid colored lines are the indicated emission profiles from the Quintuplet-labeled tonsil section and gray areas define the

band pass filters used. Profiles with the same color indicate colocalization. Scale bar is 50 mm. MIB-1 is a Ki-67 equivalent in formalin-fixed, paraffin-embedded tissue. (Reprinted by permission from Macmillan Publishers Ltd: Modern Pathology (Fountaine, Wincovitch et al. 2006) copyright 2006)

CHAPTER 3

Significance and Specific Aims

3.1 Significance of research

Cancer is a major public health problem. About 1,529,560 new cancer cases are expected to be diagnosed and about 569,490 Americans are expected to die of cancer in 2010 (American Cancer Society 2010). The 5-year survival from the most common cancers affecting people living in the United States has changed little in the last two decades (Ozols, Herbst et al. 2007). Although novel molecular-targeted agents made progress in the last decade and offer some hope in cancer treatment, clinical experience of targeted therapies is still at an early stage but more treatment options will be available in the future. PARAs are potent and selective tumor cell killers that are progressing to phase II clinical trials (Wiley, Schooley et al. 1995; Pitti, Marsters et al. 1996; Duiker, Mom et al. 2006). Because of the specificity of targeted therapy, selecting the most appropriate study patients based on the tumoral expression of the drug target is important for diseasedirected randomized clinical studies. Otherwise these trials may fail to correctly assess the usefulness of a truly effective new molecularly-targeted therapy (de Bono and Rowinsky 2002). Either DR4 or DR5 must be present on the cell surface to transduce the TRAIL signal. Evaluation of tumor cell surface DR4 and DR5 expression could be potentially useful predictors of TRAIL sensitivity (Ashkenazi and Dixit 1998; Ashkenazi and Dixit 1999; Zhang, Franco et al. 1999; Kim, Fisher et al. 2000; Ozoren, Kim et al. 2000; Ozawa, Friess et al. 2001; Shin, Kim et al. 2001; LeBlanc and Ashkenazi 2003; Jin, McDonald et al. 2004; Ma, Zhang et al. 2005; Khanbolooki, Nawrocki et al. 2006; Finnberg, Klein-Szanto et al. 2008; Grosse-Wilde, Voloshanenko et al. 2008). The

synergistic effect of chemotherapy in combination with TRAIL may result from chemotherapy-mediated increase in the expression of DR4 and DR5 (Gibson, Over et al. 2000). Pre-treatment with cisplatin or taxane followed by HGS-ETR1 or HGS-ETR2 resulted in significant higher cytotoxic effects as compared to the reverse sequence (Gong, Yang et al. 2006; Belyanskaya, Marti et al. 2007). Molecular imaging may be exploitable to optimize patient selection and sequence timing through telling how much DR4 and DR5 are expressed on the tumor cell surface and how long after pre-treatment DR4 and DR5 increased to peak. In vivo imaging can provide much more information than a tissue biopsy due to tumor heterogeneity and the effects of the tumor bulk and microenvironment in vivo. A requirement for documented presence of the target has been included in product labeling of about 70% of targeted oncology drugs approved by the FDA in recent years. So targeted oncology drugs are mainly used in patients who have the target in excised or biopsied tumor tissue. But the response rate is still relatively low (e.g. 10~14% for Avastin and Erbitux in patient with metastatic colon cancer) (Nunn 2008). One of the explanations is the extreme heterogeneity of cancer - not only every patient is different but also cancers can be different even within the same patient (Shah, Mehra et al. 2004; Torres, Ribeiro et al. 2007). Molecular imaging holds the promise to dramatically improve the targeted therapy response rate by characterizing every primary/metastatic cancer (Nunn 2008). The presence of DR4 and DR5 on the tumor cell surface may not be sufficient to predict sensitivity to PARAs if TRAIL signaling is impaired. General therapeutic response markers will improve prediction of therapeutic response, especially in combination with targeted antiproliferative therapies. We hypothesize molecularly targeted imaging of death receptors will be efficient in

predicting PARA therapeutic response and that proliferation imaging may favorably complement the death receptor target imaging in case of an impaired TRAIL signaling pathway. This multimodality molecular imaging strategy will offer great opportunities for rational selection of targeted therapy and earlier modifications to avoid progression of a nonresponsive tumor (Misek, Imafuku et al. 2004; Sokolov, Nida et al. 2007).

3.2 Specific Aims

Cancer is the second leading cause of death in the US. As previously mentioned, cancer is often associated with insufficient apoptosis. An important part of the immune response that suppresses cancer are the apoptosis-inducing receptors including the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors - death receptor 4 (DR4) and DR5 which suppresses not only cancer but also metastases. As mentioned in the introduction, TRAIL and DR4/DR5 agonistic monoclonal antibodies e.g. HGS-ETR1/HGS-ETR2 as a single agent or in combined regimens in preclinical and early clinical trials have shown significant potential for further development. It is crucial to predict the sensitivity of TRAIL based treatment for individualized patient treatment in the age of "personalized medicine" and for selecting patients most likely to benefit from this kind of therapy in future clinical trials thereby speeding the drug development process. This is especially important because cell death defects are a major mechanism by which cancer evade the immune system as well as chemotherapy in general. Biomarkers at the level of gene transcription and gene translation are in general highly sensitive but require sophisticated biostatistical interpretation and tissue and/or body-fluid sampling. Body-fluid analyses provide only information at the time point of sampling and average signal of output from all lesions. Biopsies characterize only the tissues that were biopsied and are prone to sampling errors in particular when analyzing tremendously heterogeneous cancers (Jin, McDonald et al. 2004; Shah, Mehra et al. 2004; Rudin, Rausch et al. 2005; Torres, Ribeiro et al. 2007). Ignoring or neglecting molecular tumor heterogeneity in clinical trials may fail to detect a truly effective new therapy or a realistic appraisal of efficacy (de Bono and Rowinsky 2002). In comparison, imaging approaches will yield spatially and temporally resolved information in a noninvasive manner. One of the major limitations in current imaging strategies is that several months often must go by before recognizing a failed or ineffective therapy. The overall goal of this dissertation is to develop efficient multimodality molecular imaging methods to predict proapoptotic anticancer therapy response before or at the very early stage of treatment. We hypothesize that the cell surface expression level of proapoptotic TRAIL receptors can predict the proapoptotic targeted therapeutic response following TRAILreceptor targeted therapy and that FLT uptake by tumor cells that correlates with cell proliferation can noninvasively predict proapoptotic response if the pathway is intact, and coupled with a significant decrease in FLT-PET tumor uptake after initiation of treatment might predict the anticancer therapeutic response. In order to test the validity of the hypothesis, the specific aims for this thesis are as follows:

3.2.1 Aim 1. Image proapoptotic receptor agonist binding to proapoptotic receptors *in vitro* and *in vivo* and correlate it with proapoptotic therapeutic response.

Aim 1.1. Characterize cancer cell lines and image the binding of TRAIL and HGS-ETR1/2 to DR4/5 *in vitro* and correlate it to death induction. Evaluate DR4 and DR5 expression level of cancer cell lines by Western Blotting and flow cytometry. Assess apoptosis before and after TRAIL and HGS-ETR1/2 treatment by Western Blotting, flow cytometry and short-term colony assay. Label TRAIL and HGS-ETR1/2 with NIR dye and image binding to death receptors using different cancer cell lines. Correlate the binding ability of TRAIL and HGS-ETR1/2 to DR4/5 with their proapoptotic level using cultured cells.

Aim 1.2. Image the binding of TRAIL and HGS-ETR1/2 to DR4/5 *in vivo* and correlate it with therapeutic response. Tumor xenografts were grown subcutaneously in immunodeficient mice. Labeled TRAIL and HGS-ETR1/2 were injected *intravenously* (IV). Image TRAIL and HGS-ETR1/2 binding to death receptors *in vivo*. Label Annexin V with NIR dye. Image Annexin V binding to PS on the outer plasma membrane leaflet of apoptotic cells shortly after treated with TRAIL. Tumors were harvested for immunohistochemical examinations. Correlate the binding ability of TRAIL and HGS-ETR1/2 with their proapoptotic level *in vivo*.

3.2.2 Aim 2. Image the specific marker of cellular proliferation shortly after the beginning of treatment with TRAIL and/or Sorafenib and correlate it to proapoptotic therapeutic response.

Aim 2.1. Demonstrate *n*on-*s*mall *c*ell *l*ung *c*ancer (NSCLC) uptake of ¹⁸F-3'-*f*luoro-3'deoxy-*L*-*t*hymidine (FLT) in xenograft mouse model. TRAIL and Sorafenib resistant and sensitive cell lines were characterized *in vitro*.

Aim 2.2. Assess changes in FLT-*p*ositron *e*mission *t*omography (PET) tumor uptake shortly after the beginning of treatment with TRAIL and/or Sorafenib and correlate with the tumor histological response as measured by Ki67 nuclear staining, *t*erminal dUTPbiotin deoxynucleotidyl transferase *n*ick-*e*nd *l*abeling (TUNEL) staining and H & E

CHAPTER 4

Prediction of proapoptotic anticancer therapeutic response based on cell death target visualization and death ligand-receptor interaction

4.1 Abstract

Tumor growth is often associated with insufficient apoptosis. Proapoptotic targeted therapeutics hold great promise for treatment of many cancers. The Tumor Necrosis Factor (TNF)-Related Apoptosis-Inducing Ligand (TRAIL) and its proapoptotic receptors death receptor 4 (DR4) and DR5 agonistic monoclonal antibodies are being developed as potent targeted therapeutics because they predominantly kill cancer cells while sparing normal cells. A challenge to targeted therapeutics is the selection of patients who are most likely to benefit from targeted drugs because of the heterogeneity of cancer. Molecular imaging may be useful in targeted drug development by assessing the target expression and drug-target interaction, and predicting therapeutic response. We hypothesize that cell surface expression level of DR4/5 can predict the proapoptotic targeted therapeutic response hence visualization of proapoptotic receptors and binding of their agonists to proapoptotic receptors may noninvasively predict proapoptotic response if the pathway downstream is intact. The goal of this proof of concept study is to develop an optical molecular imaging strategy to predict proapoptotic anticancer therapy response before or at the very early stage of treatment. We labeled TRAIL and DR5 agonistic monoclonal antibody HGS-ETR2 (Lexatumumab, TRM-2) with a near-infrared dye and imaged the labeled TRAIL/HGS-ETR2 binding to its receptors on cultured human colorectal carcinoma HCT116 (TRAIL sensitive) and HT29 (TRAIL resistant) cells and non-small cell lung cancer (NSCLC) H460 (TRAIL sensitive) as well as engineered

H460-DR5-298 cells expressing a TRAIL decoy thereby making them TRAIL resistnant, and tumor xenografts derived from these cells. Imaging of cells and tumor-bearing animals was conducted with near infrared fluorescence imagers. Apoptosis in cells was assessed by western blots of PARP-cleavage and flow cytometry of subG1 content. Apoptosis in tumors was evaluated by imaging of near-infrared dye-labeled Annexin V and tumor tissue activated caspase-3 staining. Both *in vitro* and *in vivo* studies showed that imaging of death inducing ligand–receptor interaction was consistent with the apoptosis readout. Thus TRAIL sensitive tumors that express TRAIL receptors were predicted to undergo cell death following treatment whereas tumors lacking TRAIL receptor expression were shown to be TRAIL resistant. *In vivo* molecular imaging of TRAIL receptor expression correlated with response to TRAIL therapy and an apoptotic response *in vivo*.

Keywords: near infrared molecular imaging; TRAIL; DR4; DR5; proapoptotic receptor agonists; therapeutic response

4.2 Introduction

Cancer is the second most common cause of death in the US (American Cancer Society 2010). The 5-year relative survival rate for all cancers diagnosed between 1995 and 2001 is 65% (Ozols, Herbst et al. 2007). Emerging targeted therapeutics offer opportunities for improving survival and response rates in hard-to-treat cancers. Tumor growth is often associated with insufficient apoptosis. The *t*umor necrosis factor-*r*elated *a*poptosis-*i*nducing *l*igand (TRAIL), a type II transmembrane protein of *t*umor *n*ecrosis *f*actor (TNF)

family, is one of the most powerful proapoptotic targeted biotherapeutic agents (Wiley, Schooley et al. 1995; Pitti, Marsters et al. 1996). TRAIL induces apoptosis in a wide variety of tumor cells with minimal to no effect on most normal cells. Preclinical studies have shown that systemic administration of TRAIL could slow the growth and, in some cases, induce regression of tumor xenografts and does not lead to appreciable toxicity in rodents or primates (Kelley and Ashkenazi 2004). Five distinct TRAIL receptors have been identified: death receptor 4 (DR4, TRAIL-R1) (Pan, O'Rourke et al. 1997), DR5 (TRAIL-R2) (Walczak, Degli-Esposti et al. 1997; Wu, Burns et al. 1997), Decoy receptor 1 (DcR1, TRAIL-R3) (Sheridan, Marsters et al. 1997), DcR2 (TRAIL-R4) (Pan, Ni et al. 1997) and Osteoprotegerin (OPG) (Emery, McDonnell et al. 1998). DR4 and DR5 are type I membrane proteins containing two extracellular cysteine-rich domains (CRD) and a conserved cytoplasmatic death domain (DD) signaling apoptosis. The stronger expression of DR4 and DR5 in neoplastic cells than in normal cells accompanied by a higher degree of apoptosis suggests a possible functional role for these receptors in apoptosis induction in neoplastic colorectal cells (Koornstra, Kleibeuker et al. 2003). DcR1 lacks transmembrane cytoplasmatic and domains and is glycosylphosphatidylinositol (GPI) linked to the cell surface. DcR2 contains a truncated nonfunctional DD. A potential decoy function of DcR1 and DcR2 has only been observed in overexperssion experiments. No differences were found between tumor and normal cells regarding DcR1 and DcR2 expression. Experiments under physiological expression conditions remain to be shown (Koornstra, Kleibeuker et al. 2003). OPG is a soluble secreted protein also known to bind to another member of the TNF family, RANKL. OPG has very low affinity for TRAIL at physiological temperature. Although apoptosis

depends on the expression of one or both of the death domain containing receptors DR4 and/or DR5, resistance to TRAIL-induced apoptosis does not correlate with the expression of the "decoy" receptors (Truneh, Sharma et al. 2000). TRAIL induces apoptosis through binding to proapoptotic receptors DR4 and DR5. Binding of TRAIL to the extracellular domain of the receptor trimer triggers the formation of the deathinducing signaling complex (DISC) by recruiting Fas-associated death domain (FADD), cysteine-dependent, aspartate-specific proteases (Caspases) -8, -10 and the FLICEinhibitory protein (FLIP), followed by activation of caspase-8, which in type I cells leads to activation of caspase-3 and rapid apoptosis (extrinsic pathway). In type II cells, insufficient activation of caspase-8 requires amplification of the apoptotic signal through cleavage of the BH3-only protein Bid and activation of the intrinsic pathway to finally induce apoptosis. Activated Bid induces oligomerization of proapoptotic proteins Bax and Bak, resulting in the release of both cytochrome c and second mitochondria-derived activator of caspases/ direct IAP binding protein with low PI (Smac/DIABLO) from the mitochondria to block the caspase-3 inhibitory effect of the X-linked inhibitor of apoptosis proteins (XIAP) and subsequent activation of caspase 9, which leads to the activation of caspase 3 and subsequently apoptosis (Scaffidi, Fulda et al. 1998). For type II cells, blockade of the mitochondrial intrinsic pathway may render resistance of TRAIL (Ozoren and El-Deiry 2002). HGS-ETR1 (Mapatumumab) and HGS-ETR2 (Lexatumumab, Human Genome Sciences) are fully humanized agonistic monoclonal antibodies with high affinity and specificity for DR4 and DR5 respectively. Proof of concept has been demonstrated that agonistic antibodies against DR4/DR5 have antitumor activity by inducing tumor cell death *in vitro* and *in vivo* and this killing was

mediated through the activation of both extrinsic and intrinsic pathways (Pukac, Kanakaraj et al. 2005; Sarantopoulos, Wakelee et al. 2005; Marini, Denzinger et al. 2006; Zeng, Wu et al. 2006). Just like TRAIL, DR4/DR5 agonistic antibody is a potent antitumor agent either used alone or in combination with other therapeutics. Moreover agonistic antibodies against DR4/DR5 may have enhanced therapeutic potential due to a prolonged half-life *in vivo* and the lack of binding to decoy receptors.

Several phase I and II clinical trials have been completed or initiated with TRAIL as well as agonistic monoclonal antibodies against DR4/DR5 as a selective killer of tumor cells. TRAIL receptor targeted therapeutics as a single agent or a combined regimen in preclinical and early clinical trials has shown significant improvements in response rates and less toxicity than conventional "non-selective" cytotoxic therapies which lack specificity for tumor cells. But cancer is genetically diverse (Jin, McDonald et al. 2004; Shah, Mehra et al. 2004; Torres, Ribeiro et al. 2007). It is crucial to predict the sensitivity of TRAIL based treatment for individualized patient treatment in the age of "personalized medicine" and for selecting patients most likely to benefit from this kind of therapy thereby speeding the clinical trials. Selecting the most appropriate study patients for disease-directed randomized clinical studies is vital to ensure that the maximal usefulness of targeted therapies is correctly assessed. Ignoring or neglecting molecular tumor heterogeneity in clinical trials may fail to detect a truly effective new therapy (de Bono and Rowinsky 2002). Since serum analyses provide only an average signal of output from all lesions and biopsies characterize only the tissues that were biopsied, molecular imaging provides a great opportunity to characterize the tremendous heterogeneity of cancer. Molecular imaging is a technique that uses molecules or generates signals from

molecules to image specific molecular pathways *in vivo*, particularly those that are key targets in disease processes (Weissleder and Mahmood 2001). Optical imaging is emerging as a powerful new modality of molecular imaging to detect, diagnose, characterize, or monitor tumors before and after therapeutic intervention. Comparing with computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET) and single-photon emission computed tomography (SPECT), optical imaging is real time, robust, cost-effective, rapid, easy to use, and can be readily applied to studying disease processes and biology in vivo. Biologic tissues are opaque to most wavelengths of light. Thus only small animals and structures close to the surface can be imaged with most visible light. However many biologic tissues are relatively transparent to near infrared (NIR), which enables imaging structures of 5 to 10 cm from the surface. Translational medical research applications of optical imaging to clinical medicine include the potential for breast imaging and other organs when the detection devices are positioned near the fluorescent probe, such as through endoscopy and intraoperative scanning (Shah, Gibbs et al. 2005; Cerussi, Hsiang et al. 2007; Torigian, Huang et al. 2007; Veiseh, Gabikian et al. 2007; Luker and Luker 2008). Previous studies using Cy 5.5-Annexin V as a NIR probe to noninvasively image tumor apoptosis *in vivo* post radiotherapy and chemotherapy demonstrated excellent fluorochrome stability and temporal advantage over radionuclide imaging (Reynolds, Troy et al. 1999; Petrovsky, Schellenberger et al. 2003).

In this study, we show that optical molecular imaging of a therapeutic ligand and its target in tumor cells and xenografts could serve as a potential predictor of the proapoptotic treatment response. The proapoptotic receptors DR4 and DR5 must be

present on the cell surface to transduce the TRAIL agonist triggered apoptosis-inducing signal. Deficiency of DR4/DR5 expression or failed receptor trafficking to cell surface confers tumor cell resistance to TRAIL-induced apoptosis (Ashkenazi and Dixit 1998; Zhang, Franco et al. 1999; Kim, Fisher et al. 2000; Ozoren, Fisher et al. 2000; Ozoren, Kim et al. 2000; Jin, McDonald et al. 2004). In the screen of small interfering RNA (siRNA) library for genes regulating TRAIL sensitivity, the signal recognition particle (SRP) complex was identified and related to cell surface levels of DR4 as well as DR4mediated apoptosis (Ren, Wagner et al. 2004). Reintroducing wild type DR4 into DR4 null TRAIL resisting FaDu cells leads to apoptosis and restores TRAIL sensitivity (Ozoren, Fisher et al. 2000). Here we show that NIR imaging of TRAIL agonists binding to death receptors could predict the TRAIL based therapeutic response if the pathway downstream is intact. This technique has important implications for improving predictability and efficiency of personalized treatment and for speeding drug development by stratifying subjects, differentiating responders from non-responders and informing selection of candidates for clinical testing.

4.3 Materials and Methods

4.3.1 Cell lines and culture

The human colorectal carcinoma HCT116 (TRAIL sensitive) and HT29 (TRAIL resistant) cells and *n*on-*s*mall *c*ell *l*ung *c*ancer (NSCLC) H460 (TRAIL sensitive) cells were obtained from the *A*merican *Type Culture Collection* (ATCC). Considering artificial overexpression of death-domain-containing death receptors causes cell death in a ligand-independent manner (because of the spontaneous self-aggregation of death domains when death receptors are expressed above physiological levels), which may interfere with the

assay, we designed a DR5 mutant that loses the ability to induce apoptosis after transfection into cells (Tschopp, Martinon et al. 1999). H460 DR5-298-EGFP cells were generated by inserting the open reading frame of human DR5 without the C-terminal death domain into pEGFP-N1 vector (Clontech) to express a fused protein with EGFP, transfecting the construct to H460 cells with Lipofectamine 2000 (Invitrogen) and selecting with G418 for 10 days. Then a cell clone with DR5-EGFP expression was isolated and passaged in RPMI 1640 with 10% of *f*etal *b*ovine *s*erum (FBS) and 1% *pen*icillin and *strep*tomycin (Pen/Strep). HCT116 and HT29 cells were grown in McCoy's 5A media containing 10% FBS and 1% Pen/Strep. H460 and H460 DR5-298-EGFP cells were grown in RPMI 1640 media with 10% FBS and 1% Pen/Strep at 37°C and 5% CO₂.

4.3.2 Production of recombinant His₆-tagged soluble human TRAIL

The extracellular portion (aa 95~281) of the human TRAIL cDNA was amplified and cloned into pQE80L (Qiagen) after digestion with BamHI and HindIII to generate the His₆-tagged *soluble human* TRAIL expression plasmid. Recombinant His₆-tagged soluble human TRAIL was expressed in competent *E. coli* DH10B cells. A single colony of recombinant *E. coli* was picked from a streaked LB agar plate supplemented with 100 μ g/ml ampicillin and inoculated in 30 ml of LB broth supplemented with 100 μ g/ml ampicillin in a 250 ml flask. The culture was grown overnight (~16 hours) in a 37°C shaking incubator. The overnight seed culture was diluted 100-fold into 2 liter of LB broth with 100 μ g/ml ampicillin and incubated for 3 hours at 37°C in a shaking incubator. 0.5 mM *I*sopropyl-1-*t*hio- β -D-galactopyranoside (IPTG, Fisher Scientific) was added to induce recombinant protein expression, and bacterial cells were incubated for another 3

hours at 37°C in a shaking incubator. Cells were spun down and resuspended in 2% of initial culture volume binding buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole (a histidine analogue), and 10 mM β -mercaptoethanol). Lysozyme was added at 1 mg/ml and cells were sonicated in ice and spun down to collect the supernatant. Ni-NTA (nickel–nitrilotriacetic acid; Qiagen) agarose beads were added to bind recombinant His₆-tagged soluble human TRAIL. After washing with buffer containing 50 mM imidazole, recombinant His₆-tagged soluble human TRAIL was eluted with elution buffer containing 250 mM imidazole. The purity of the eluted protein was confirmed by SDS-PAGE and Coomassie Blue gel staining. Eluted protein was dialyzed against PBS. The concentration of the protein was checked using a Bio-Rad protein assay (Kim, Kim et al. 2004).

4.3.3 Production of recombinant polyhistidine-tagged soluble Annexin V

pProEx.Htb.Annexin V plasmid was kindly provided by Professor Seamus J. Martin at the Trinity College in Ireland (Brumatti, Sheridan et al. 2008). Then following the procedure of production of recombinant His₆-tagged soluble human TRAIL to express and purify recombinant polyhistidine-tagged soluble Annexin V.

4.3.4 Label TRAIL, Annexin V and HGS-ETR2 with IRDye800CW

The *IRDye800CW* protein labeling kit (Low MW) was obtained from Li-Cor Biosciences. The labeling procedure was performed by following the manufacturer's protocol. In brief, protein concentration was adjusted to 1mg/ml and the pH value was raised to 8.5 after buffer exchange. The protein was warmed to 20~25°C. 0.5mg of dye were dissolved in 25 μ l of ultra pure water and mixed thoroughly by vortexing. An appropriate amount of dye was added to the protein according to the *m*olecular *w*eight (MW) of the protein: Dye volume (μ l) = 233.2/MW. The mixture was left to react for 2 hours at 20°C in the dark. The conjugate was separated from the free dye using a desalting column equilibrated with PBS. The dye/protein ratio and protein concentration were determined spectrophotometrically:

$$Dye / \Pr otein = \frac{A_{780}}{\varepsilon_{Dye}} \div \frac{A_{280} - (0.03 \times A_{780})}{\varepsilon_{\Pr otein}}$$

 $ProteinConc.(mg/ml) = \frac{A_{280} - (0.03 \times A_{780})}{\varepsilon_{Protein}} \times MW_{Protein} \times DilutionFactor$

Where 0.03 is a correction factor for the absorbance of IRDye 800CW at 280 nm (equal to 3.0% of its absorbance at 780 nm), $\varepsilon_{Dye}=270,000 \text{ M}^{-1}\text{cm}^{-1}$, $\varepsilon_{TRAIL}=27,515 \text{ M}^{-1}\text{cm}^{-1}$, $\varepsilon_{Annexin} = 21,500 \text{ M}^{-1}\text{cm}^{-1}$, $\varepsilon_{HGS-ETR2}=210,000 \text{ M}^{-1}\text{cm}^{-1}$, $MW_{TRAIL}=23,000$, $MW_{Annexin} = 23,000$, $MW_{HGS-ETR2}=143,600$. ε_{TRAIL} was predicted with following equation: (Pace, Vajdos et al. 1995)

$$\varepsilon(280)(M^{-1}cm^{-1}) = (\#Trp) \times 5500 + (\#Tyr) \times 1490 + (\#Cys) \times 125$$

4.3.5 In cell western assay with labeled TRAIL and HGS-ETR2: Cells were plated in 96-well black plates with clear bottom and cultured to confluency. In a separate 96-well plate devoid of cells, 1:2 serial dilutions of IRDye 800CW labeled TRAIL and HGS-ETR2 were prepared from well 3 to 12 and medium only was added to the first two wells. Media was removed from cells by inversion. 50 μ l labeled protein was transferred from the dilutions preparing plate into the cell-containing plate quickly. Cells were incubated at room temperature for 5 minutes. Cells were immediately fixed by adding 150 μ l of fresh 3.7% formaldehyde. Plate was covered with foil and incubated at room temperature for 5 minutes. Cells were washed four times with 200 μ l of 0.1% Triton X-100 for 5 minutes with shaking to permeabilize the cells. 150 μ l/well LI-COR Odyssey

blocking buffer (LI-COR Biosciences) with 0.1% Tween-20 were added and incubated for 1.5 hours at RT with shaking. TO-PRO-3 is a cell stain that can be used to normalize the signal for cell number in each well. The stain was diluted 1:5000 in LI-COR Odyssey Blocking Buffer. 50 μ l of LI-COR Odyssey Blocking Buffer + 0.1% Tween-20 was added to Wells 1 and 2. These wells served as controls for any potential background due to the stain. 50 μ L of diluted TO-PRO-3 were added to well 2 to 12 and cells were incubated for 1 hour with shaking. Cells were washed four times with 0.1% Tween-20 for 5 minutes at room temperature. The plate was scanned simultaneously at 700 nm and 800 nm using the Odyssey Infrared Imaging System (LI-COR Biosciences). A medium scan quality, 169 μ m resolution, 3.5 mm focus offset, and an intensity setting of 5 for the 700 channel and 7 for the 800 nm channel were used.

4.3.6 Near-infrared imaging of labeled TRAIL, HGS-ETR2 and Annexin V in xenografts

Healthy female nu/nu mice aged 4–5 weeks from Charles River Breeding Laboratories were inoculated subcutaneously with 2×10^{6} HCT116, HT29, H460 and H460 DR5-298-EGFP cells in each flank or shoulder. Cells were suspended in 50% Matrigel[®] matrix (Fisher Scientific) in PBS. Mouse body weight and tumor dimensions were periodically measured. Tumor volumes were estimated using the following equation (Euhus, Hudd et al. 1986; Tomayko and Reynolds 1989):

$$TumorVolume = \frac{1}{2} \times length \times width^2$$

Tumors were established for 10 days (the tumor volume of $\sim 150 \text{mm}^3$). Mice were anesthetized with an *i*ntraperitoneal (IP) injection of 2 mg ketamine/0.3 mg xylazine. One group of mice was treated with TRAIL (100 µg/mouse) via IV injection and 6 hours later

they received an IV injection of labeled Annexin V. The other two groups received IV injection of labeled TRAIL and labeled HGS-ETR2 without treatment. All mice were prescanned and postscanned from 2 minutes to 9 days after beacon injections. The imager used is the Pearl Impulse *in vivo* fluorescent imaging system (LI-COR Biosciences).

4.3.7 Immunohistochemistry

As described above, when HCT116, HT29, H460 and H460 DR5-298-EGFP tumors were established for 10 days (the tumor volume of ~150mm³), mice were treated with vehicle (PBS) or TRAIL (100 μ g/mouse) via *i*ntravenous (IV) injection daily for 5 days consecutively. Mice were sacrificed and tumors were excised and weighted. Tumors were cut into halves and fixed with 4% paraformaldehyde solution for 24 hours, then washed and dehydrated in 70% ethanol. Tumors were paraffin embedded and sectioned into 5 μ m thick slices and mounted onto slides for analysis of active caspase-3 (an *in situ* marker for apoptosis) and Ki67 (a nuclear antigen strongly correlated with active cellular proliferation).

4.3.8 Western blot analysis

As described previously (Jin, McDonald et al. 2004), cells were treated as desired, rinsed with PBS and lysed directly in 24-well plates by NuPAGE[®] LDS Sample Buffer (Invitrogen). Lysates were boiled for 10 minutes and then resolved by SDS-PAGE and polypeptides were electrophoretically transferred to polyvinylidene difluoride membranes (Millipore Corp.). Membranes were blocked with 10% non-fat milk for 1 hour at room temperature. Membranes were incubated overnight at 4°C with primary antibodies: rabbit anti-human DR4 (BD Biosciences), rabbit anti-human DR5 (C20) (Santa Cruz Biotechnology), rabbit anti-human PARP (Cell Signaling)

and mouse anti-human Ran (BD Biosciences). Membranes were washed six times for 2, 2, 2, 5, 15, 5 minutes respectively, and incubated with species-specific secondary antibodies horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Amersham Biosciences) for 1 hour at room temperature. Blots were washed six times for 2, 2, 2, 5, 30, 5 minutes respectively, and developed by the Enhanced Chemiluminescence system (Amersham Biosciences).

4.3.9 Flow Cytometric Detection of DR4/DR5 Surface Expression

The experiments were performed as described previously (Zhang, Franco et al. 2000; Jin, McDonald et al. 2004). In brief, one million cells were rinsed and detached with cell dissociation buffer (Invitrogen). Cells were spun down and washed with 1% FBS in PBS, and then resuspended in 100 µl 1% FBS in PBS containing 1 µg primary antibody against DR4 or DR5 (Alexis Biochemicals) or a nonspecific mouse IgG1 antibody (CALTAG Laboratories) as a control. After staining on ice for 60 min, cells were washed once with 1% FBS in PBS and incubated with 1µg secondary antibody Alexa Fluor[®] 647-R-Phycoerythrin Goat anti-Mouse IgG (Invitrogen) at 4°C for 45 min in the dark. Cells were analyzed on an Epics Elite flow cytometer (Beckman Coulter).

4.3.10 Sub-G1 DNA content analysis

Cells were plated in 6-well plates and treated as needed. The floating cells were collected and the adherent cells were trypsinized. Cells were spun down and washed with 1% FBS in PBS, then fixed with cold 70% ethanol. After cells were spun down and washed with 1% FBS in PBS, 0.5 ml phosphate-citric acid buffer (192 ml 0.2 M Na₂HPO₄+8 ml 0.1 M citric acid; pH 7.8) was added to elute small DNA fragments. Cells were stained with 50μg/ml *P*ropidium *i*odide (PI, Sigma) and RNase at room temperature for 30 min and then analyzed on an Epics Elite flow cytometer (Beckman Coulter).

4.3.11 Short term colony survival assay

A total of 2×10^4 cells/well were seeded in 24 well plate and cultured for a week, then cells were treated with 0.4 µg/ml TRAIL or 1 µg/ml HGS-ETR1/2. After 4 hours, the medium was removed and cells were rinsed with 500µl PBS. 500µl of methanol was added to fix the cells for 15 min at room temperature. Methanol was removed and 500µl Coomassie Blue was added, and cells were incubated for 5 min. Cells were rinsed with 500µl PBS and then scanned for visualization and image preservation.

4.3.12 Statistical Analysis

Quantitative data was analyzed, including t-test, linear regression and correlation, using the EXCEL Spreadsheet Data Analysis ToolPak (Microsoft Office).

4.4 **Results and Discussion**

4.4.1 TRAIL is a more powerful apoptosis inducer than agonistic monoclonal antibodies

Human colorectal carcinoma cell line HCT116 and non-small cell lung cancer cell line H460 are sensitive to TRAIL treatment while HT29 is quite resistant to TRAIL (Ozoren and El-Deiry 2002; Ricci, Kim et al. 2007). We engineered H460 cells to overexpress truncated DR5 (aa1-298) without death-domain to avoid ligand-independent apoptosis. We confirmed the sensitivity of HCT116 and H460 cell lines and resistance of HT29 and H460-DR5-298-EGFP cell lines to TRAIL and HGS-ETR1/2 treatment with western blotting of cleavage of PARP (Figure 4.1B and Figure 4.9B), flow cytometry of subG1 content (Figure 4.3) and short term colony survival assay (Figure 4.4). HCT116 cells
were most sensitive to TRAIL, next to HGS-ETR2 and least to HGS-ETR1 as shown in Figure 4.1B. H460 cells were similarly more sensitive to TRAIL than to HGS-ETR2 as shown in Figure 4.9B. These results support the idea that TRAIL is a more powerful apoptotic agent than agonistic monoclonal antibodies since TRAIL can bind to both DR4 and DR5.

4.4.2 TRAIL sensitive cancer cells have higher DR4 and DR5 expression on the cell surface as compared to TRAIL resistant HT29 cells

Either DR4 or DR5 must be present on the cell surface in order to respond to PARA treatment. Earlier studies showed that tumor cell surface DR4 and DR5 expression could predict TRAIL sensitivity (Ashkenazi and Dixit 1998; Ashkenazi and Dixit 1999; Zhang, Franco et al. 1999; Kim, Fisher et al. 2000; Ozoren, Kim et al. 2000; Ozawa, Friess et al. 2001; Shin, Kim et al. 2001; LeBlanc and Ashkenazi 2003; Jin, McDonald et al. 2004; Ma, Zhang et al. 2005; Khanbolooki, Nawrocki et al. 2006; Finnberg, Klein-Szanto et al. 2008; Grosse-Wilde, Voloshanenko et al. 2008). The total and cell surface expression levels of DR4 and DR5 were evaluated with Western Blotting (Figure 4.1A and Figure 4.9C) and flow cytometry (Figure 4.2). Both total and cell surface expression levels of DR4 and DR5 of HCT116 were more than 10 times higher than that of HT29 as shown in Figure 4.1A.

4.4.3 TRAIL resistant cancer cells may have high DR4 and/or DR5 expression on cell surface if downstream signaling is impaired

As shown in the schematic representation of DR5 structure (Figure 4.9A), H460-DR5-298-EGFP cells overexpressed truncated DR5 (1-298) without death domain. H460-DR5-298-EGFP and H460 cell lines have the same amount of full length DR5 as probed with antibodies raised against a peptide mapping at the carboxyl terminal of human DR5 (Figure 4.9C upper panel). But the total DR5 expression level of H460-DR5-298-EGFP soared to more than 80 times higher than that of H460 when probed against residues surrounding cysteine 248 of isoform 1 of human DR5 (Figure 4.9C lower panel). Taken together these results support that overexpression of a death receptor devoid of a death domain has a decoy function. But this is a different story from physiological expression conditions – usually no differences were found between tumor and normal cells regarding DcR1 and DcR2 expression (Koornstra, Kleibeuker et al. 2003). Resistance to TRAIL-induced apoptosis does not correlate with the expression of the "decoy" receptors (Truneh, Sharma et al. 2000). This study can partially explain why some cancer cells with reasonable expression of DR4 and DR5 are resistant to TRAIL induced apoptosis.

4.4.4 DR4 and DR5 cell surface expression level can be visualized with near infrared dye labeled PARAs (TRAIL and agonistic monoclonal antibodies)

Recombinant soluble human TRAIL was expressed and purified. The purity was confirmed with SDS–PAGE gel. Figure 4.6B is the Coomassie Blue stained SDS–PAGE gel of samples taken before (uninduced), or after (induced), induction of protein expression, supernatant after Ni-NTA agarose beads binding (Supernatant), each wash fraction (Wash 1, Wash 2, Wash3, Wash4) and each elution fraction (Elution 1, Elution 2, Elution 3, Elution 4). The ~23 kDa TRAIL band appeared after induction of expression but vanished in supernatant after Ni-NTA agarose beads binding. The yield and purity were adequate as 200 ml culture was found to yield more than 1 milligram recombinant soluble human TRAIL protein apparently free from contaminating protein.

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Recombinant soluble human TRAIL and HGS-ETR2 were labeled with IRDye 800CW. The absorption spectrum of IRDye 800CW labeled HGS-ETR2 (Figure 4.5 B) is comparable to the free dye absorption spectrum (Figure 4.5 A) – the peak falls around 800 nm which is amenable to detection by the Odyssey and Pearl Infrared Imaging System 800 nm channel image.

IRDye 800CW labeled recombinant soluble human TRAIL was checked with SDS-PAGE gel. Figure 4.6C is the 800 nm channel image of IRDye 800CW labeled recombinant soluble human TRAIL SDS-PAGE gel collected by the Odyssey Infrared Imaging System. Besides the monomer form of TRAIL (23 KDa), there are dimer and even trimer forms of TRAIL (65 kDa). This is in agreement with gel filtration chromatography analyzed data (Kim, Kim et al. 2004). Adding a polyhistidine tag to TRAIL provides a simple means of isolating TRAIL from other contaminating bacterial proteins in a single-step affinity purification procedure. The polyhistidine tag is smaller than most other affinity tags (e.g. GST or maltose-binding protein) and is thus less likely to interfere with the function of TRAIL (i.e., the ability to bind to DR4/5). But His₆tagged TRAIL is not optimized for zinc content which is crucial for stability. Thus it tends to form insoluble aggregates that may cause apoptosis in normal cells in preclinical studies (Lawrence, Shahrokh et al. 2001; Kim, Kim et al. 2004). We did observe insoluble aggregates of His₆-tagged TRAIL and increased the sodium chloride concentration of dialysis buffer to 300 mM to reduce insoluble aggregates. SubG1 analysis demonstrated that labeled TRAIL and HGS-ETR2 retain their apoptosis inducing function (data not shown here). So the binding ability of labeled TRAIL and HGS-ETR2 to death receptors is not compromised by conjugation.

To assess TRAIL receptor cell surface expression level of HCT116 and HT29 cells, incell western assay was performed with IRDye 800CW labeled TRAIL. HCT116 cells gave a much brighter signal than HT29 cells in the 800 nm channel (Figure 4.7 A). To normalize well-to-well variation in cell number, TO-PRO-3 staining (specific for nuclear counterstaining) was scanned in the 700nm channel. The normalized binding ability of labeled TRAIL to HCT116 was 3 times as that to HT29 (Figure 4.7 B). To estimate DR5 cell surface expression level of HCT116, HT29, H460 and H460-DR5-298-EGFP cells, IRDye 800CW labeled HGS-ETR2 was applied to an in-cell Western assay (Figure 4.8 A and Figure 4.10 A). The normalized binding ability of IRDye 800CW labeled HGS-ETR2 to HCT116 was 3 times as that to HT29 while binding to H460-DR5-298-EGFP cells was 5 times as that to H460 (Figure 4.8 B and Figure 4.10 B). These differences were not as prominent as observed in Western blotting (Figure 4.1 A and Figure 4.9 C) but still evident. The binding ability of labeled TRAIL and HGS-ETR2 to death receptors *in vitro* was strongly correlated with the cell surface death receptor expression level.

4.4.5 PARA imaging correlation with TRAIL induced apoptosis imaging in vivo

To image cancer cell death receptor expression level *in vivo*, IRDye 800CW labeled TRAIL and HGS-ETR2 were injected IV into HCT116, HT29, H460 and H460-DR5-298-EGFP xenograft-bearing mice and imaged with the Pearl near-infrared imager. IRDye 800CW labeled HGS-ETR2 signal of HCT116 was twice as that of HT29 (Figure 4.11 A). We observed the IRDye 800CW labeled HGS-ETR2 signal of H460-DR5-298-EGFP was five times as that of H460 (Figure 4.13 B). The IRDye 800CW labeled TRAIL signal of H460-DR5-298-EGFP was three times as that of H460. (Figure 4.13 A) *In vivo*

imaging of DR4/5 expression level was consistent with Western blotting, flow cytometry and in-cell western assay findings.

Annexin V binds to phosphatidylserine (PS) on the outer plasma membrane leaflet of apoptotic cells and is widely used for the detection of apoptosis (Belhocine, Steinmetz et al. 2002; Petrovsky, Schellenberger et al. 2003; Mandl, Mari et al. 2004; Ntziachristos, Schellenberger et al. 2004; Dicker, Kim et al. 2005; Henery, George et al. 2008). To image TRAIL induced apoptosis in vivo, polyhistidine-tagged Annexin V was expressed and purified following the TRAIL production protocol. The purity of recombinant soluble polyhistidine-tagged Annexin V was confirmed with SDS-PAGE gel (Figure 4.6 A). Annexin V was labeled with IRDye 800CW for imaging apoptosis. HCT116, HT29, H460 and H460-DR5-298-EGFP xenografts bearing mice were treated with TRAIL intravenously. Six hours later IRDye 800CW labeled Annexin V was injected IV and the 800 nm channel image was collected with the Pearl imager. IRDye 800CW labeled Annexin V signal of HCT116 was twice that of HT29 (Figure 4.11 B). The IRDye 800CW labeled Annexin V signal of H460-DR5-298-EGFP was three times as that of H460 (Figure 4.13 C). In vivo image signal intensity of TRAIL induced apoptosis was consistent with PARP cleavage, SubG1 content and short term colony survival assay (Figure 4.1, 4.3, 4.4 and 4.9).

The DR5 expression level image with IRDye 800CW labeled HGS-ETR2 (Figure 4.11 A) and TRAIL induced apoptosis image with IRDye 800CW labeled Annexin V (Figure 4.11 B) of HCT116 and HT29 showed a strong positive correlation (Figure 4.11 C). By contrast, the DR5 expression level image with IRDye 800CW labeled HGS-ETR2 (Figure 4.13 B) and TRAIL induced apoptosis image with IRDye 800CW labeled

Annexin V (Figure 4.13 C) of H460 and H460-DR5-298-EGFP showed a strong negative correlation (Figure 4.13 D middle and right plots). Even DR4/5 expression level image with more general IRDye 800CW labeled TRAIL (Figure 4.13 A) and TRAIL induced apoptosis image with IRDye 800CW labeled Annexin V (Figure 4.13 C) of H460 and H460-DR5-298-EGFP showed a strong negative correlation (Figure 4.13 D left and right plots).

Of note, the Annexin V signal lasted for several days – there was still strong signal 4 days and even 8 days post IV injection of IRDye 800CW labeled Annexin V (Figure 4.12 A and B). This gives the benefit of a flexible imaging schedule.

4.4.6 PARA imaging correlation with immunohistochemistry post TRAIL treatment

The body weight of both TRAIL treated and control group mice bearing HCT116, HT29, H460 and H460 DR5-298-EGFP xenografts did not change significantly (Figure 4.14). This is consistent with prior reports that TRAIL is safe in both preclinical and clinical trials (Herbst, Mendelson et al. 2006; Ling, Herbst et al. 2006). Mouse tumor size is usually evaluated based on tumor dimensions measured with calipers. Comparing tumor volume with excised tumor weight gave very strong positive correlations (Figure 4.15). So we can track tumor volume to estimate tumor weight in preclinical treatment trials. Tumor shrinkage is one of the treatment response markers. Comparing tumor size before and post TRAIL treatment, the mean values of HCT116 and H460 tumor volume were less than those of HT29 and H460-DR5-298-EGFP but the variations were too big so the differences were not significant.

That HCT116 is sensitive to and HT29 is resistant to TRAIL induced apoptosis was confirmed with tumor active Caspase-3 staining (Figure 4.16). TRAIL induced extensive

apoptosis in HCT116 tumors but not in HT29 tumors. This result was evident with labeled Annexin V imaging post TRAIL treatment (Figure 4.11 B) and DR5 expression level imaging of HCT116 and HT29 tumors using labeled HGS-ETR2 (Figure 4.11 A). Optical molecular imaging that can directly assess molecular targets shows great promise for predicting targeted therapeutic response. On the other hand, tumor Ki67 staining revealed that TRAIL inhibited cellular proliferation in H460 tumors but not in H460-DR5-298-EGFP tumors (Figure 4.17). Like labeled Annexin V imaging (Figure 4.13 C), this was negatively correlated with the targeted death receptor expression imaging with labeled TRAIL and HGS-ETR2 (Figure 4.13 A and B). Different from wild-type DR5, the existence or overexpression of DR5-298 did not improve TRAIL induced apoptosis but increased the dominant-negative effect – in this case DR5-298 inhibited TRAIL signaling through the DR4 receptor by competing for ligand binding (Bin, Thorburn et al. 2007).

4.5 Conclusion

Emerging targeted therapeutics hold great promise for the treatment of human cancer. However there are numerous challenges for selecting patients who are most likely to benefit from targeted drugs. Molecular imaging can be useful to characterize target protein expression, drug-target interactions and tumor response, hence stratify patients with regard to the presence or absence of a drug target and provide tests to distinguish rapidly between responders and nonresponders to a drug. Engagement of cell surface death receptors by TRAIL is the initial step in the induction of intracellular death signaling pathways and apoptosis. DR4/DR5 is essential for TRAIL and agonist antibody induced apoptosis. This proof-of-concept study supports that imaging of DR4/DR5 and

binding of TRAIL and agonist antibodies to DR4/DR5 is feasible and can predict proapoptotic anticancer therapeutic response. We conclude that TRAIL imaging is safe in preclinical treatment trial and TRAIL is a more powerful apoptosis inducer than agonistic monoclonal antibodies. Cell surface DR4 and DR5 expression level was correlated with sensitivity to TRAIL induced apoptosis and could be imaged with near infrared dye labeled TRAIL and agonistic monoclonal antibodies. Both in vitro and in vivo studies demonstrated that imaging PARAs binding to their targets was well correlated with proapoptotic anticancer therapeutic response when the TRAIL signaling pathway was intact. Overexpression of truncated DR5 reversed H460 sensitivity to TRAIL induced apoptosis and turned a strong positive correlation between imaging PARAs binding to death receptors and PARA treatment response to a strong negative correlation. To pursue a more general molecular imaging marker that can predict anticancer therapeutic response even when the signaling pathway is impaired, we explored imaging of phosphatidylserine-sensing Annexin V post TRAIL treatment and demonstrated additional correlations between target imaging and cell death in vivo.



Figure 4.1 The correlation between death receptors DR4/5 expression levels of HCT116 and HT29 cells and their response to death receptor agonists TRAIL and HGS-ETR1/2 treatment. (A) DR4 and DR5 expression levels of HCT116 and HT29 were determined by western blotting. Ran serves as loading control. (B) 4 hours post-treatment, apoptosis of HCT116 and HT29 induced by different dosage of TRAIL and HGS-ETR1/2 as indicated was assessed for PARP cleavage by western blotting. Ran serves as loading control.



Figure 4.2 HCT116 and HT29 cell surface expression levels of DR4 and DR5 were analyzed by flow cytometry. Primary antibody is control mouse IgG1 or Alexis mouse monoclonal antibody to human DR4 (HS101) or monoclonal antibody to human DR5 (HS201). Secondary antibody is Alexa Fluor® 647-R-Phycoerythrin Goat anti-Mouse IgG (H+L).



Figure 4.3 SubG1 content of HCT116 and HT29 was analyzed by flow cytometry before and 4 hours post 50 ng/ml or 200 ng/ml TRAIL treatment.



Figure 4.4 Short term colony survival assay of HCT116 and HT29: 4 hours post treatment with 0.4 μ g/ml TRAIL or 1 μ g/ml HGS-ETR1/2 TRAIL, survival colonies of HCT116 and HT29 cells were stained with Coomassie Blue in 24-well culture plate.



Figure 4.5 The absorption spectra of IRDye 800CW (A) and IRDye 800CW labeled HGS-ETR2 (B).



Figure 4.6 Purified recombinant soluble Annexin V and TRAIL and labeled TRAIL.

(A) SDS–PAGE gel of purified recombinant soluble Annexin V; (B) SDS–PAGE gel of samples taken at various stages of the process of bacterial expression and purification of recombinant TRAIL. Samples were taken from culture before (Uninduced), or after (Induced), induction of protein expression, supernatant after Ni-NTA agarose beads binding (Supernatant), each wash fraction (Wash 1, Wash 2, Wash3, Wash4) and each elution fraction (Elution 1, Elution 2, Elution 3, Elution 4). Note the appearance of the ~23 kDa TRAIL band after induction of expression. (C) SDS–PAGE gel of IRDye 800CW labeled TRAIL imaged at 800 nm with the Odyssey Infrared Imaging System.



Figure 4.7 Imaging TRAIL receptor expression level of HCT116 and HT29 with IRDye 800CW labeled TRAIL using in-cell Western assay. (A) Plate setup for in-cell western assay and 800-channel image. (B) Normalization using TO-PRO-3 is detected in the 700-channel. The graph represents normalized quantitative data demonstrating the differentiation of HCT116 and HT29 binding ability to TRAIL.



Figure 4.8 Imaging DR5 expression level of HCT116 and HT29 with IRDye 800CW labeled DR5 antibody HGS-ETR2 using in-cell western assay. (A) Plate setup for incell western assay and 800-channel image. (B) Normalization using TO-PRO-3 is detected in the 700-channel. The graph represents normalized quantitative data demonstrating the differentiation of HCT116 and HT29 binding ability to DR5 antibody HGS-ETR2.



Figure 4.9 The correlation between truncated DR5 expression level of H460 cells and their response to death receptor agonists TRAIL and HGS-ETR2. (A) Schematic representation of DR5 structure: H460-DR5-298-EGFP cells overexpressed truncated DR5 (1-298) without death domain. (B) Apoptosis induced by TRAIL and DR5 antibody HGS-ETR2 was assessed for PARP cleavage by western blotting. Ran serves as loading control. (C) Full length DR5 and all DR5 expression levels were probed with antibodies raised against a peptide mapping at the carboxyl terminal of human DR5 and a peptide corresponding to residues surrounding cysteine 248 of isoform 1 of human DR5 respectively. Shorter exposure signals are shown on the right. Ran serves as loading control.



Figure 4.10 Imaging TRAIL receptor expression level of H460 and H460-DR5-298-EGFP with IRDye 800CW labeled TRAIL using in-cell western assay. (A) Plate setup for in-cell western assay and 800-channel image. (B) Normalization using TO-PRO-3 is detected in the 700-channel. The graph represents normalized quantitative data demonstrating the differentiation of H460 and H460-DR5-298-EGFP binding ability to TRAIL.



Figure 4.11 Imaging DR5 expression level and TRAIL induced apoptosis *in vivo*. (A) Imaging of labeled HGS-ETR2 binding to HCT116 and HT29 *in vivo*. (B) Imaging of apoptosis induced by TRAIL with IRDye 800CW labeled Annexin V. (C) Quantitative comparison of DR5 expression level of (A) and TRAIL induced apoptosis (B). The histograms show the labeled DR5 antibody signal intensity is correlated with that of labeled Annexin V.



- Animals were imaged 4 and 8 Days post IV Annexin V injection



Figure 4.12 Imaging of apoptosis induced by TRAIL with IRDye 800CW labeled Annexin V on day 4 (A) and day 8 (B) post labeled Annexin V intravenous injection.



Figure 4.13 Imaging DR5 expression level and TRAIL induced apoptosis. (A) Imaging of IRDye 800CW labeled TRAIL binding to H460 and H460-DR5-298-EGFP *in vivo*. (B) Imaging of IRDye 800CW labeled DR5 antibody HGS-ETR2 binding to H460 and H460-DR5-298-EGFP *in vivo*. (C) Imaging of apoptosis induced by TRAIL with IRDye 800CW labeled Annexin V. (D) Quantitative comparison of labeled TRAIL signal intensity (A), labeled DR5 antibody HGS-ETR2 signal intensity (B) and labeled Annexin V signal intensity (C). The histograms show the truncated DR5 overexpressed H460-DR5-298-EGFP has stronger binding ability to TRAIL and DR5 antibody but less apoptosis.



Figure 4.14 The body weight change of mice bearing HCT116, HT29, H460 and H460 DR5-298-EGFP xenografts after treated with TRAIL ($100\mu g$ /mouse) via IV injection daily for 5 days consecutively.



Figure 4.15 Correlation of tumor volume and tumor weight. Tumor volume of mouse subcutaneous tumors HCT116, HT29, H460 and H460 DR5-298-EGFP were estimated by measuring tumor dimensions. Excised tumors were weighted. (A) Mouse tumor volume and weight were plotted in the same chart. Correlation analysis reported Correlation Coefficient 0.888. (B) Regression analysis of mouse tumor volume and weight reported Coefficient of Determination 0.7879.



Figure 4.16 Active Caspase-3 staining of control and TRAIL treated HCT116 and HT29 tumors. (A) Original scan of control HCT116 tumor; (B) High-power view of control HCT116 tumor (original scan ×40); (C) Original scan of TRAIL treated HCT116 tumor; (D) High-power view of TRAIL treated HCT116 tumor (original scan ×40); (E) Original scan of control HT29 tumor; (F) High-power view of control HT29 tumor (original scan ×40); (G) Original scan of TRAIL treated HT29 tumor; (H) High-power view of TRAIL treated HT29 tumor (original scan ×40).



Figure 4.17 Ki67 staining of control and TRAIL treated H460 and H460-DR5-298-EGFP tumors. (A) Original scan of control H460 tumor; (B) High-power view of control H460 tumor (original scan ×40); (C) Original scan of TRAIL treated H460 tumor; (D) High-power view of TRAIL treated H460 tumor (original scan ×40); (E) Original scan of control H460-DR5-298-EGFP tumor; (F) High-power view of control H460-DR5-298-EGFP tumor; (G) Original scan of TRAIL treated H460-DR5-298-EGFP tumor (original scan ×40); (G) Original scan of TRAIL treated H460-DR5-298-EGFP tumor; (H) High-power view of TRAIL treated H460-DR5-298-EGFP tumor; (original scan ×40).

CHAPTER 5

FLT-PET as an early marker of therapeutic response in non-small cell lung cancer:

the value of xenograft animal modeling in imaging clinical trial design

This chapter was submitted to the Journal of Nuclear Medicine and co-first authored with Sharyn I. Katz, and co-authored with Wenge Wang, Thomas Ferrara, Patrick A. Mayes, Charles Smith and Wafik S. El-Deiry.

5.1 Abstract

Rationale: ¹⁸F-3'-*f*luoro-3'-deoxy-*L*-*t*hymidine (¹⁸F-FLT), a novel *p*ositron *e*mission *t*omography (PET) tracer of proliferation, is being developed as an early therapeutic response indicator. In this manuscript, we examine variables to be considered in assessing treatment-related changes in tumor uptake of FLT and discuss the role of animal modeling in clinical trial design.

Materials and Methods: The sensitivities of the human non-small cell lung cancer cell line CALU-6 to TRAIL and Sorafenib was confirmed *in vitro* with cell viability assays and subG₁ FACS analysis. Three animal trials were conducted with CALU-6 tumorbearing Nu/Nu Mouse (Crl:NU-Foxn1^{nu}) xenografts with daily treatment with TRAIL (200 μ g IV)/ Sorafenib (30 mg/kg IP) therapeutic durations of 11-19 days. In the first trial of 8 mice, tumor changes in FLT uptake were assessed at 96 hrs of treatment. This experiment was repeated with 25 mice, this time using tumors too large to treat successfully, with changes in tumor FLT uptake assessed at 72 hrs of treatment. Finally, in a trial of 30 mice, daily TRAIL/Sorafenib therapy was compared to drug vehicle and weekly doses of cisplatin (IP)/docetaxel (IP) with tumor treatment related changes in FLT uptake measured at 72 hours. In all trials, tumor response was also assessed with external tumor caliper measurement and histology.

Results: Xenografted CALU-6 tumors responded to daily combination therapy TRAIL IV/ Sorafenib IP as determined by tumor caliper measurements, decreased accumulation of AngioSense, a blood-pooling optical imaging agent, decreased Ki-67 nuclear staining, increased TUNEL staining and tumor hyalinization. There were significant decreases in tumor uptake of FLT at 72 hours of TRAIL/Sorafenib therapy. Reductions in tumor FLT uptake were also seen after therapy with cisplatin/docetaxel but were however were very modest given the changes in tumor volume and apoptosis seen at end of therapy.

Conclusions: Early changes in FLT-PET may not only predict success of a chemotherapeutic regimen but also determine superiority of one treatment regimen over another. The timing of last dose administration of chemotherapy may affect the scale of the FLT response. Further trials are needed to fully assess this variable.

Keywords: FLT, PET, response, lung, cancer

5.2 Introduction

Lung cancer is the leading cause of cancer-related death world-wide with 1.3 million deaths/year (*WHO*, 2009 *http://www.who.int/mediacentre/factsheets/fs297/en/index.html*), the majority of whom are diagnosed at advanced stage with less than one-year survival. With targeted therapy taking center stage for lung cancer therapeutics, imaging methods tailored to measuring changes in targeted endpoints are needed especially given that the currently accepted response criteria, New Response Evaluation Criteria in Solid Tumors RECIST 1.1. (Eisenhauer, Therasse et al. 2009) was formulated based on conventional therapy and imaging. For example the *tyrosine kinase inhibitors* (TKIs) erlotinib and

gefitinib, may manifest response with stable disease rather than with significant tumor regression as measured by RECIST1.1.

Furthermore, individual patient therapy and pharmaceutical development would benefit greatly if imaging of therapeutic response could take place in days to weeks rather than months as currently done with CT. Additionally, functional imaging agents designed to measure components of specific molecular pathways and targets allow for a better understanding of tumor biology and heterogeneity. Earlier response feedback would allow for an earlier switch to a more effective therapy and optimize the process of drug development. For all these reasons, functional imaging biomarkers of tumor response are now of intense interest. One such promising new biomarker of response to therapy is ¹⁸F-3'-*f*luoro-3'-deoxy-*L*-*t*hymidine (¹⁸F-FLT), a novel PET radiotracer and marker of tumor proliferation.

FLT is an analogue of thymidine and has been validated as a marker of proliferation in numerous recent publications. This tracer has already shown promise (Bading and Shields 2008; Larson and Schoder 2009), both in animal models (Barthel, Cleij et al. 2003; Leyton, Latigo et al. 2005; Buck, Kratochwil et al. 2007; Chao 2007; Molthoff, Klabbers et al. 2007; Graf, Herrmann et al. 2008; Kim, Lee et al. 2008; Debucquoy, Devos et al. 2009; Lawrence, Vanderhoek et al. 2009; Pantaleo, Landuzzi et al. 2009) and clinical trials(Sugiyama, Sakahara et al. 2004; Pio, Park et al. 2006; Chen, Delaloye et al. 2007; Herrmann, Ott et al. 2007; Herrmann, Wieder et al. 2007; Kenny, Coombes et al. 2007; Sohn, Yang et al. 2008; Ullrich, Zander et al. 2008; Been, Hoekstra et al. 2009; Brockenbrough, Morihara et al. 2009; Everitt, Hicks et al. 2009), as an early indicator of therapeutic response potentially as early as 24-48 hours after chemotherapeutic

administration. FLT is metabolized via the DNA salvage pathway and is trapped within the cell by phosphorylation mediated through thymidine kinase 1 (TK1). The expression and activity of TK1 is tightly regulated by cell cycle control mechanisms (Hu and Chang 2007; Ke, Hu et al. 2007; Brockenbrough, Morihara et al. 2009; Munch-Petersen 2009). Since thymidine is not a component of RNA, the FLT trapped and concentrated in the cell reflects the product of DNA synthesis only, although FLT is not actually incorporated into the DNA as are other thymidine analogues such as tritiated thymidine. A challenge to emerging chemotherapy for lung cancer is the prevalence of resistance to tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), an innate stimulator of programmed cell death, either at the onset or during the course of therapy. In an earlier publication from our laboratory we described the sensitization of TRAIL resistant human cancer cell lines CALU-6 and HT29 following pre-incubation with the multi-targeted tyrosine kinase inhibitor, Sorafenib, in vitro and in vivo, an effect mediated via suppression of MCL-1L and c-IAP2 (Ricci, Kim et al. 2007). Both TRAIL pathway therapeutics (Spierings, de Vries et al. 2003; Voortman, Resende et al. 2007; Greco, Bonomi et al. 2008; Stegehuis, de Wilt et al. 2010), primarily explored via proapoptotic receptor agonists (PARAs) and Sorafenib (Carter, Chen et al. 2007; Gridelli, Maione et al. 2007; Blumenschein, Gatzemeier et al. 2009; Takezawa, Okamoto et al. 2009) have demonstrated potential in the management of non-small cell lung cancer primarily as part of a multi-drug regimen. In particular, Sorafenib has been very promising in a growing number of malignancies and is currently FDA approved for unresectable hepatocellular carcinoma and renal malignancy.

With the proliferation of potential therapeutic drug combinations, an efficient means to triage drug efficacy would be invaluable not only for selection of drug therapies for a particular tumor phenotype but also to expedite the discovery process. In this chapter, we evaluate a validated xenograft mouse model of non-small cell lung cancer as a tool for comparing drug therapy regimens employing emerging imaging agents of drug response. Cisplatin and docetaxel combination therapy, a conventional regimen for treatment of lung cancer, was compared to combination therapy with TRAIL and Sorafenib. Correlation was made between early changes in tumor uptake of FLT-PET and morphologic tumor response to therapy, assessed with external tumor caliper measurement and histology. The optical imaging agent AngioSense (Montet, Ntziachristos et al. 2005; Binstadt, Patel et al. 2006; Kelly, Waterman et al. 2006), which is a blood pooling agent and thus a measure of tumor vascularity, was also explored as a specific endpoint to targeted therapy with Sorafenib.

5.3 Materials and Methods

5.3.1 Cell lines and cell culture: The human cell line utilized for *in vitro* studies was CALU-6 which was newly purchased from *A*merican *Type Culture Collection* (ATCC, Manasas, VA). Care was taken to limit the number of passages to less than 20 to avoid inadvertent *in vitro* selection. When not in use, cells were stored in liquid nitrogen in 10% DMSO/ 90% culture medium.

Cells were maintained in a humidified sterile environment with 5% CO_2 , 37°C RPMI medium supplemented with 10% heat-inactivated fetal calf serum with 1% penicillin and streptomycin (CM). Passage of cell lines was performed at 1:5 dilution after detachment using sterile 0.5% trypsin-EDTA solution.

5.3.2 Chemotherapeutics: Flag-TRAIL was synthesized via plasmid expression and purification and stored as a 1 mg/ml stock in dPBS at -20°C. Sorafenib was synthesized in powder, free base form for use in our laboratory. Sorafenib was dissolved in 100% DSMO and stored at -20°C as a concentrated stock for *in vitro* studies. Sorafenib was dissolved in a Cremaphor EL solution (Sigma-Aldrich, St. Louis, MO), made freshly for each daily dose for *in vivo* studies. Cisplatin powder was purchased from (Sigma-Aldrich, St. Louis, MO) and prepared freshly in dPBS prior to each injection. Docetaxel 10 mg/ml solution was obtained from the University of Pennsylvania Abramson Cancer Center pharmacy and diluted in a 13% EtOH solution.

5.3.3 *In vitro* cell death assays: Cells were harvested from culture with 0.5% trypsin-EDTA and washed with the CM then plated at 0.6×10^6 /well in a sterile 6-well plate. Cells were then incubated at 37°C, 5% CO₂ for 18-24 hours to allow attachment of cells to the wells. Sorafenib (diluted in CM from 32mM stock in DMSO) was then added to the appropriate wells for a total of 4 ml CM and cells were allowed to incubate with Sorafenib for 16-22 hours. For comparison, cells were also treated for 4 hours of TRAIL therapy with and without pre-incubation with Sorafenib, Flag-TRAIL (diluted in CM from a 1 mg/ml stock) was added to the appropriate wells at either 50 ng/ml or 100 ng/ml. Culture medium for wells, not containing Sorafenib, was treated with 0.1% DMSO to control for the possible effects of DMSO on cell viability. After incubations with the chemotherapeutics, cells were washed with cold sterile dPBS then fixed with 10% methanol/10% acetic acid solution for 10 minutes. These fixed adherent cells were then stained with 2 ml of 0.25% Coomassie Blue solution for 3 minutes followed by gentle rinse with distilled water and then allowed to air-dry. **5.3.4 Propidium Iodide FACS:** After incubations with chemotherapeutics in a 60 mm tissue culture dish, supernatants and adherent cells were removed from the dish surface using 0.5% trypsin-EDTA, and washed with 5ml cold dPBS. Cell pellets were then resuspended in 5 ml cold 75% EtOH and allowed to fix from overnight to 1 month at 4°C. On the day prior to FACS analysis, the EtOH was removed and cells were washed with 5ml cold dPBS, permeabilized with 0.5ml phosphate-citric acid buffer. (192ml 0.2M Na₂HPO₄+8ml 0.1M citric acid; pH should be ~7.8) for 5 minutes, then resuspended in ~300µl PBS/FBS/PI/RNase solution (300µl 1×PBS /1%FBS+30µl 0.5mg/ml PI+5.5µl USB RNase) and allowed to incubate overnight at 4°C. The cells were then sorted by FACS the next day and the subG₁ fraction measured.

5.3.5 Xenograft formation Prior to the use of animal models, protocol approval was obtained by the IACUC at the University of Pennsylvania. 2 month old female Nu/Nu mice (Crl:NU-Foxn1^{nu}) (Charles River Laboratory) were injected subcutaneously in each flank with 2×10^6 cells suspended in a 50% sterile, endotoxin-free Matrigel solution. After therapy/imaging completion, mice were euthanized with an intraperitoneal injection of 6 mg ketamine/0.9 mg xylazine followed by cervical dislocation.

5.3.6 *In vivo* therapeutic trials: Following xenograft formation, FLT-PET or FDG-PET was performed prior to initiation of chemotherapy. Sorafenib was administered at a dose of 30 mg/kg diluted in a Cremaphor EL/13% EtOH vehicle solution and injected IP daily for 11-19 days. TRAIL was diluted in PBS and injected by tail vein at 100 µg/mouse daily. Cisplatin was diluted in PBS vehicle and injected IP at 3mg/kg daily. Docetaxel was obtained freshly diluted from the Abramson Cancer Center pharmacy in a 13% ETOH vehicle solution and injected IP at 7.5 mg/kg daily. Both cisplatin and docetaxel were dosed weekly.

A total of three *in vivo* trials were conducted in order to assess the dynamics of FLT-PET uptake in several therapeutic settings (Figure 5.1). The first trial was a pilot study of 8 CALU-6 tumor-bearing xenografted mice, half of which received TRAIL/Sorafenib therapy for 15 days and the other 4 were untreated (no vehicle). FLT-PET imaging was performed, along with micro CT, just prior to therapy initiation and at day 4 of daily therapy. A period of 24 hours was allowed without chemotherapy prior to FLT-PET in order to avoid drug-drug interactions.

Before euthanasia, all mice were imaged with the AngioSense (VisEn Medical, Inc., Bedford, MA) optical imaging agent, a specific marker for angiogenesis.

The second trial was a cohort of 24 CALU-6 tumor-bearing mice divided into 2 groups, half of which received TRAIL/Sorafenib and the other received no treatment (no vehicle). The tumors were allowed to grow to approximately 300 mm³ prior to therapy in order to assess changes in FLT-PET uptake in a tumor sensitive to therapy but refractory to successful treatment due to size. These mice received TRAIL/Sorafenib daily in the manner described above for a total of 2 weeks. FLT-PET was performed before and at 3 days of daily therapy. A period of 24 hours was allowed without chemotherapy prior to FLT-PET in order to avoid drug-drug interactions. After euthanasia, tumors were excised and preserved for histology in a 4% para-formaldehyde solution.

Finally, in the 3rd trial, tumors were allowed to grow to 200 mm³ to ensure large enough tumors to image. A total of 25 mice were employed in this cohort, 8 in the TRAIL/Sorafenib group, 8 in the cisplatin/docetaxel group, 5 in no treatment/no imaging

group, and 4 in vehicle only group (2 in Cremaphor EL vs 2 in PBS). In this trial, TRAIL/Sorafenib, which was dosed daily, was given 1 hour prior to beginning the FLT-PET imaging session. Cisplatin/docetaxel, which was dosed weekly, was given 3 days prior to the post-therapy FLT imaging.

5.3.7 **PET imaging and image interpretation:** Tumors were allowed to grow until they reached 150-200 mm³ by caliper measurements utilizing the formula $TumorVolume = \frac{1}{2} \times length \times width^2$. FLT and FDG radiotracers were synthesized in the

in the PET Cyclotron Facility at the University of Pennsylvania. After anesthesia with inhaled isoflurane in O₂ (3% induction, 1.5% maintenance), mice were injected with 200-250 mCi of FLT or FDG then allowed to recover from the anesthesia and given a 60 minute uptake time to allow radiotracer to accumulate in the tumor. At 60 minutes, the mice were again anesthetized and imaged for a 15 min static acquisition on the small animal PET scanner (A-PET, built in collaboration with Philips Medical Systems) located at the University of Pennsylvania. This system has very high spatial resolution (approx 2 mm), operates exclusively as a fully 3D instrument with very high sensitivity and large field-of-view of 12 cm in both axial and transverse directions. Images were reconstructed with the manufacturer software and tumor volumes of FLT and FDG were quantitated with the freely available Amide 3D software (*A*mide's a *M*edical *I*mage *D*ata *E*xaminer (AMIDE), LG Software Innovations), which allows for multiplanar analysis of the tumor volume for ROI (region of interest) placement and statistical data analysis including the mean and max standardized uptake value (SUV) for FLT and FDG in the assigned ROI.

5.3.8 Optical imaging: At 48 hours prior to sacrifice during experiment 1, each mouse was injected with 100 mg of AngioSense 750 IV by tail vein and a time course of

imaging (pre, 20 minutes, 6 hours, 24 hours and 48 hours) was acquired on the Maestro multispectral imaging system (CRI, Woburn MA), with the deep red filter set (excitation: 670-710nm; acquisition mono: 780nm; acquisition cube: 730 to 900nm), with mice anesthetized with an intraperitoneal injection of 2 mg ketamine/0.3 mg xylazine. The AngioSense signal was measured and unmixed from background autofluorescence using the CRI post-processing software. Immediately after euthanasia, the tumors were excised and AngioSense signal measured directly from the tumor on the IVIS system using ICG filter set (excitation: 710-760 nm; emission: 810-875 nm).

5.3.9 Histological Analysis After euthanasia, tumors were promptly excised, weighed and immersed in a 4% paraformaldehyde solution and allowed to incubate overnight at 4°C. The tissues were then removed and placed in a solution of 70% ethanol for from 24 hours to 2 weeks, maintained at 4°C until paraffin embedding, sectioned into 5 mm thick slices and mounted onto slides. Slides were stained for Ki67, a nuclear antigen strongly correlated with active cellular proliferation, and the *t*erminal deoxynucleotidyl transferase d*U*TP *n*ick *end l*abeling (TUNEL), a method for detecting DNA fragmentation generated during apoptosis by labeling the terminal end of nucleic acids.

5.3.10 Statistical Analysis: Quantitative data was analyzed, including T-test, linear regression and correlation, using the EXCEL Spreadsheet Data ToolPak (Microsoft Office).

5.4 **RESULTS**

The human non-small cell lung cancer line CALU-6 is sensitive to Sorafenib and TRAIL combination therapy *in vitro* and *in vivo*. To establish a xenograft model of the spectrum of therapeutic response in human lung cancer, we explored whether CALU-6 cells

demonstrate TRAIL resistance and synergistic response to therapy with TRAIL/Sorafenib both *in vitro*, as previously observed (Ricci, Kim et al. 2007), and *in vivo*. CALU-6 is a commercially available human non-small cell lung cancer line previously characterized as TRAIL resistant and modestly sensitive to Sorafenib. We again demonstrated TRAIL resistance, modest sensitivity to Sorafenib and synergistic, dose-dependent cell killing achieved with combination Sorafenib/TRAIL best accomplished with Sorafenib pretreatment followed by the addition of TRAIL. CALU-6 sensitivities were confirmed using brightfield microscopy (Figure 5.2), subG₁ FACS analysis by propidium iodide staining and cell killing assays with Coomassie blue stain.

5.4.1 FLT-PET is a sensitive predictor of response of tumor to therapy at 72 hours of treatment. ¹⁸F-FLT uptake by CALU-6 was validated by FLT-PET/CT imaging of a xenograft mouse at 60 minutes post 209µCi FLT IV injection (Figure 5.3). With CALU-6 response to TRAIL/Sorafenib therapy established *in vitro*, we then examined CALU-6 tumor response to therapy with TRAIL/Sorafenib in an Nu/Nu Mouse (Crl:NU-Foxn1^{nu}) model. In addition, we assessed changes in tumor accumulation of FLT at 72-96 hours of combination therapy with TRAIL and Sorafenib in order to explore changes in FLT uptake as a predictor of tumor morphological and histological response to therapy. In order to explore the sensitivity of FLT-PET to detect actual tumor response to therapy, rather than only inherent tumor cell sensitivity to the TRAIL/Sorafenib regimen, changes in tumor accumulation of FLT were also examined in the setting of failure of CALU-6 xenograft tumor treatment with TRAIL/Sorafenib accomplished by starting therapy with xenograft tumors that were too large to treat successfully.
In the first *in vivo* experiment, the changes in tumor accumulation of FLT observed at 96 hours of daily TRAIL/Sorafenib therapy were correlated with changes in tumor volume and histology measured at the termination of therapy. The tumor volume change over the 15 day treatment period was 52% (TRAIL/Sorafenib) and 206% (untreated) respectively and was predicted by the change in the max SUV for FLT (FLTmax) at 96 hours of therapy, -17% (TRAIL/Sorafenib) versus 9% (untreated) respectively (p=0.05) (Figure 5.4). Changes in the mean SUV for FLT (FLTmean) for treated and control groups did not reach statistical significance. Since Sorafenib has known anti-angiogenic activity, tumor response to therapy was also assessed, at the completion of the therapy, with the optical imaging with AngioSense, a blood-pooling agent. As expected, there was a marked decrease in tumor accumulation of AngioSense in the TRAIL/Sorafenib treatment group relative to the untreated controls (Figure 5.5).

In the second *in vivo* trial, changes in FLT uptake at 72 hours correctly predicted the failure of large CALU-6 tumors to respond to therapy with TRAIL/Sorafenib, despite the known inherent sensitivity of CALU-6 to TRAIL/Sorafenib therapy. The faster growth of the treatment group relative to the control (544% versus 314%) was attributed to allocation of larger tumors to the treatment group to avoid early termination of the experiment due to IACUC regulations. Increase in tumor accumulation of FLT at 72 hours of treatment was correlated with failure of the treatment regimen as determined by overall tumor change and histological response after 19 days of TRAIL/Sorafenib daily therapy. The average change in tumor FLTmax for control versus treated was -7% vs. 10% (p= 0.11) respectively (Figure 5.4). FDG-PET was not able to predict the failure of TRAIL/Sorafenib therapy in this trial. The average change in the max SUV for FDG

(FDGmax) for control versus treated was -20% and -10% respectively. These results again demonstrate that early changes in FLT uptake are sensitive to therapeutic efficacy and may outperform FDG-PET for this purpose.

5.4.2 Differential early changes in FLT-PET uptake predicted superiority of TRAIL/Sorafenib regimen over cisplatin/docetaxel in CALU-6 tumor-bearing **xenograft mice.** We also wished to explore whether early changes in tumor accumulation of FLT was sensitive to relative chemotherapeutic efficacy and therefore able to distinguish responses between two therapies. In the 3rd trial of TRAIL/Sorafenib therapy, a total of 25 mice were studied in the following groups: no treatment/no imaging (n=5), vehicle only (Cremaphor EL, n=2 vs PBS, n=2), TRAIL/Sorafenib (n=8) and cisplatin/docetaxel (n=8). Five mice were neither treated nor given the vehicle or imaged so as to provide a normal tumor growth trajectory for comparison. After 2 weeks of daily therapy with TRAIL/Sorafenib, changes in tumor volume and histology revealed that daily TRAIL/Sorafenib therapy was more effective at slowing tumor growth compared to cisplatin/docetaxel. This differential tumor therapeutic response was predicted by changes in tumor uptake of FLT at 72 hours (Figure 5.4 and 5.6). TRAIL/Sorafenib therapy resulted in a slowed tumor growth relative to the untreated group (21% vs.104%,p=0.00001) and vehicle Cremaphor EL group (21% vs. 53%, p=0.06) which was predicted by a significant drop in tumor accumulation of FLTmax (-30% and -17% for TRAIL/Sorafenib and vehicle Cremaphor EL respectively) (Figure 5.4). The treatment effect exerted by the Cremaphor EL vehicle, (53% growth versus 104%) for the vehicle Cremaphor EL and untreated groups respectively (p=0.02) was predicted by the decrease in FLTmax uptake (-17%) relative to baseline at 72 hours (Figure 5.4).

Cisplatin/docetaxel therapy resulted in a slowed tumor growth relative to the untreated group (54% vs.104%, p=0.008) and vehicle PBS group (54% vs. 143%, p=0.01) which was discordant with tumor accumulation of FLTmax (-2% and -12% for cisplatin/docetaxel and vehicle PBS respectively) (Figure 5.4). While the TRAIL/Sorafenib therapeutic group did show more marked slowing of tumor growth than the cisplatin/docetaxel group (21% vs. 54%, p=0.02), both groups demonstrated efficacy which was underestimated by change in tumor FLTmax accumulation in the cisplatin/docetaxel group. The FLTmean changes were correlated with the terminal changes of tumor volume as plotted in Figure 5.7.

While changes in tumor accumulation of FDG-PET at 72 hours was predictive of therapeutic efficacy of cisplatin/docetaxel and TRAIL/Sorafenib, FDG-PET was not able to discriminate between the relative efficacies of the 2 treatment groups or detect the anti-tumor effect of the Cremaphor EL vehicle.

5.4.3 Optical imaging with AngioSense demonstrated decreased vascularity of tumors associated with Sorafenib therapy. A major endpoint of targeted therapy with Sorafenib is angiogenesis. AngioSense, a near-infrared optical blood pooling imaging agent, preferentially accumulated in the vascular spaces of the CALU-6 xenograft tumors and demonstrated significantly less avidity for tumors treated with Sorafenib/TRAIL. This provides another measure of response detection in animal models of malignancy when studied for endpoints of anti-angiogenic therapy. The signal from AngioSense was imaged optimally at 48 hours after contrast administration. The decreased vascularity of the TRAIL/Sorafenib treated tumors was also easily identified *ex vivo* in white light and

with near infrared AngioSense signaling, allowing correlation without tumor repositioning (Figure 5.5).

5.5 **DISCUSSION**

In this study, we explored the sensitivity of early changes in FLT-PET to predict therapeutic efficacy in a xenograft model of *n*on-*s*mall *c*ell *l*ung *c*ancer (NSCLC). While they are imperfect models of human disease, murine xenograft models of malignancy provide a vehicle for exploration of the variables that impact FLT-PET imaging in the therapeutic setting. We chose a targeted therapeutic combination, TRAIL/Sorafenib, since such agents represent an area of intense drug development and are yet to be fully characterized with respect to therapeutic response measurement. TRAIL/Sorafenib, is a novel combination for lung cancer; however, both drugs are independently in clinical trials to assess efficacy in NSCLC (Spierings, de Vries et al. 2003; Voortman, Resende et al. 2007; Blumenschein 2008; Blumenschein, Gatzemeier et al. 2009; Stegehuis, de Wilt et al. 2010). Finally the use of TRAIL and Sorafenib in this mouse model of lung cancer produces a spectrum of response since CALU-6 is TRAIL resistant, modestly responsive to Sorafenib monotherapy and demonstrates a pronounced response with combination therapy (Ricci, Kim et al. 2007; Kim, Ricci et al. 2008).

For this study we began by confirming the sensitivity profile of CALU-6 first *in vitro* and then *in vivo* using CALU-6 grafted Nu/Nu (Crl:NU-Foxn1^{nu}) female mice. These mice demonstrated sufficiently low endogenous levels of thymidine to allow for FLT-PET analysis, a factor which can complicate the use of mouse models for FLT imaging (Mankoff, Shields et al. 2005). Daily TRAIL/Sorafenib therapy yielded significant decreases in tumor volume and increased apoptosis. This morphologic evidence of

therapeutic response to TRAIL/Sorafenib therapy strongly correlated with significant decreases in tumor uptake of FLT-PET 4 days post the initiation of daily therapy (Figure 5.4 and 5.6).

Once CALU-6 tumor responsiveness to TRAIL/Sorafenib was established *in vivo*, the sensitivity of FLT-PET to therapeutic efficacy was explored. First, since larger tumors are known to interfere with successful therapeutic response in mouse models, the size of the tumor volumes at the start of therapy was doubled to 300-350 mm³. Despite the intrinsic sensitivity of CALU-6 to TRAIL/Sorafenib, these large tumors did not respond to daily TRAIL/Sorafenib therapy (Figure 5.4). This lack of response was correctly predicted by FLT-PET at 72 hrs of daily therapy and confirmed with end of trial tumor volumes and histology. The rate of growth was higher than expected in the treated group, likely a result of larger tumors being allocated to this group to avoid early termination. This was done to ensure that both groups would remain viable for the duration of the experiment, and not sacrificed prematurely due to institutional IACUC regulations regarding allowed tumor growth. Since the goal of this experiment was to evaluate early FLT-PET responses to suboptimal therapy, rather than proving therapeutic efficacy of TRAIL/Sorafenib, this was not felt to be a significant confounder.

Finally, the efficacy of two therapeutic regimens was conducted in order to examine the sensitivity of changes in tumor accumulation of FLT to superiority of one effective regimen over another. A total of 25 mice bearing subcutaneous CALU-6 tumors were allocated to therapy with TRAIL/Sorafenib, cisplatin/docetaxel or a control group (Figure 5.5). CALU-6 sensitivity to cisplatin/docetaxel therapy in cell culture and xenograft models has been well established in the literature (Schiller and Bittner 1999; Strawn,

Kabbinavar et al. 2000; Teicher, Chen et al. 2000; Teicher, Forler et al. 2000; Miknyoczki, Jones-Bolin et al. 2003; Gourdeau, Leblond et al. 2004; Davies, Logie et al. 2007) and the therapeutic dosage and schedule was chosen accordingly. As expected, TRAIL/Sorafenib daily therapy resulted in slowed tumor growth, increased apoptosis and evidence of tumor necrosis which was again predicted by changes in tumor uptake of FLT-PET at 72 hrs of daily therapy (Figure 5.4). The accepted *in vivo* vehicle for Sorafenib, Cremaphor EL, demonstrated anti-tumor effects, a known intrinsic property (ten Tije, Verweij et al. 2003) of this vehicle. These anti-tumor effects included decreased tumor uptake of FLT-PET, slowed tumor growth and increased tumor apoptosis. The antitumor effects of Cremaphor EL vehicle alone was modest relative to that of TRAIL/Sorafenib, and while this may confound assessment of therapeutic efficacy of TRAIL/Sorafenib, it was interesting that changes in tumor uptake of FLT accumulation at 72 hrs could discriminate between the untreated, vehicle and TRAIL/Sorafenib treatment groups (Figure 5.4).

The major caveat to the conclusions from these series of studies was that the lack of significant change in FLT-PET tumor uptake in the cisplatin/docetaxel treatment group despite a moderate treatment effect evidenced by decreased tumor volumes and increased tumor apoptosis relative to controls. It is likely that this discordance between early FLT-imaging and morphological/histological response in the cisplatin/docetaxel treatment group is attributable to pharmacological properties of the drugs themselves resulting in differing drug half-life, dosing schedule, and bioavailability. We hypothesize that the timing of the dosing schedule relative to FLT imaging was responsible for the lack of observed effect of cisplatin/docetaxel on tumoral FLT uptake. The TRAIL/Sorafenib

group was dosed daily with a dose delivered 1-3 hours prior to post-treatment FLT-PET. Cisplatin/docetaxel was dosed weekly with the last dose delivered 3 days prior to post-therapeutic FLT-imaging. This 3 day delay prior to imaging that may be responsible for the inadequate effect on the post-treatment tumor accumulation of FLT. TK1, which phosphorylates and traps FLT in the cell, has fluctuating expression and activity which is tightly regulated by the cell cycle (Hu and Chang 2007). This dynamic response of proliferation, which includes chemotherapeutic insult, has been documented, although not studied in the setting of FLT-PET imaging. A systematic evaluation the relationship of chemotherapeutic pharmacokinetics and tumor proliferation is therefore needed.

Thus while robust clinical trials are ultimately needed to fully characterize drug efficacy, animal models are still valuable in the early stages of therapeutic development. The use of FLT in evaluation of drug efficacy allows for serial assessment of impact of therapy on tumor proliferation, comparison of tumor proliferation with changes in targets of therapy, and may allow use of more than one drug, drug combination or drug dosage to be examined within the same animal cohort.

5.6 CONCLUSION

In summary we have demonstrated that early changes in tumor uptake of FLT-PET are not only predictive of tumor sensitivity to a chemotherapeutic regimen, but also to subtle differences in tumor responsiveness either due to ineffective therapy or superiority of one effective treatment regimen over another. Our results highlight the need for further characterization of the relationship between therapeutic drug half-life and time of measurement of tumor accumulation of FLT.



Figure 5.1 Algorithms for the three treatment/imaging trials: (A) Pilot comparison of FLT uptake before and 4 days after combination therapy with TRAIL/Sorafenib. (B) Evaluation of the impact of increased tumor size on FLT as a predictor of response to therapy in a drug combination known to have efficacy with CALU-6.



Figure 5.1 Algorithms for the three treatment/imaging trials: (C) Comparison of TRAIL/Sorafenib and cisplatin/docetaxel, 2 therapy regimens that have efficacy and the ability of changes in tumor uptake of FLT-PET to predict response to therapy



Figure 5.2 Brightfield microscopy images of CALU-6 demonstrate sensitivities to TRAIL and Sorafenib. CALU-6 demonstrated resistance to TRAIL, modest sensitivity to Sorafenib and synergistic cell killing with combination therapy with TRAIL and Sorafenib.



Figure 5.3 FLT-PET/CT of Lung Cancer Xenografts. FLT-PET/CT was taken at 60 minutes post 209µCi FLT IV injection.



Figure 5.4 FLT is accumulated by CALU-6 mouse xenograft tumors at differing rates, depending on the therapy administered. (a) Daily TRAIL/Sorafenib therapy for 96 hours resulted in a marked drop in FLTmax tumor accumulation relative to untreated controls. (b) FLTmax response to TRAIL/Sorafenib therapy was not observed at 72 hours after daily therapy in mice with xenograft tumors too large to treat successfully. (c) Daily TRAIL/Sorafenib therapy for 72 hours resulted in an expected marked decrease in tumor accumulation of FLTmax relative to controls. The Cremaphor EL vehicle also demonstrated some anti-tumor activity as expected (d) Tumor FLTmax response at 72

hours after 1st weekly dose cisplatin/docetaxel did not demonstrate a response to therapy relative to controls.





Figure 5.5 Treatment of CALU-6 xenografts with daily TRAIL/Sorafenib for 15 days resulted in a robust therapeutic response as measured by morphological and histological measures. (a, e) Optical imaging with blood pooling agent AngioSense demonstrated marked decreased intratumoral vascularity after TRAIL/Sorafenib but not in the untreated group (b, f). Tumor excision following sacrifice revealed smaller, smoother, paler tumors in the TRAIL/Sorafenib group (c.) compared to untreated (d.). Post-treatment morphological changes correlated with histological markers of response including a robust increase in apoptosis as measured by TUNEL staining (green), in the treated (g) relative to untreated tumors (h). Blue nuclear staining represents DAPI counterstain. AngioSense *in vivo* signals were quantified (i).



Figure 5.6 Tumor FLT-PET imaging revealed response to therapy at 72 hours after treatment initiation. FLT-PET demontstrated marked suppression of tumor proliferation 72 hours after daily TRAIL/Sorafenib therapy but little response 72 hours after cisplatin/docetaxel therapy which is dosed weekly. Timing of drug dosing relative to FLT-PET imaging may affect scale detectable change in tumoral accumulation of FLT.



Figure 5.7 Xenograft uptake changes of FLT-PET predicted response to therapy at

3 Days. Liner regression of change in FLTmean uptake versus changes in tumor volume revealed significant correlations between FLTmean and changes in tumor volume.

CHAPTER 6

Summary and Future Work

6.1 Summary

Increased understanding of cancer biology has led to the development of promising targeted therapies tailored to perturb cancer-specific pathways. Inducing apoptosis and inhibiting proliferation are two of most effective ways to treat cancer. TRAIL based proapoptotic receptor agonists and the multikinase inhibitor Sorafenib studied in this dissertation are prime examples of targeted therapies - targeting apoptosis and proliferation respectively. Targeted therapeutics have shown great promise in early studies but did not produce the expected miraculous results in the clinic come out even though most patients had documented presence of the target in excised or biopsied tumor tissue. The main culprit is the extreme heterogeneity of cancer.

In this dissertation optical molecular imaging was exploited to assess DR4 and DR5 expression on the tumor cell surface. We found that DR4/5 cell surface expression level can be visualized with near infrared dye labeled TRAIL and DR4/5 agonistic monoclonal antibodies HGS-ETR1/2 *in vitro* and *in vivo*. Imaging of DR4/5 expression level is correlated with TRAIL induced apoptosis as confirmed with Western blotting of PARP cleavage, flow cytometry of subG1 DNA content, short term colony survival assay and active caspase-3 staining of tumor tissue section. TRAIL induced apoptosis can be imaged with near infrared dye labeled Annexin V *in vivo*. Images of cell surface DR4/5 expression are correlated with those of TRAIL induced apoptosis. But this correlation breaks down when TRAIL signaling is impaired. Engineered H460-DR5-298-EGFP cells have truncated DR5 overexpressed on cell surface but are resistant to TRAIL induced

apoptosis for lack of death domain. This further proved that labeled PARA imaging reported the DR4/5 levels on cell surface instead of the total expression levels. This proof-of-concept study supports that imaging of DR4/DR5 and binding of TRAIL and agonist antibodies to DR4/DR5 is feasible and can predict proapoptotic anticancer therapeutic response if TRAIL signaling is intact. The strategy we developed in this work for optical molecular imaging of therapeutic targets *in vitro* and *in vivo* with near infrared fluorescent dye labeled therapeutic agent can be extended to studies of other targeted therapeutics. Imaging apoptosis directly with phosphatidylserine-sensing Annexin V at 6 hour post TRAIL treatment is a complementary strategy that can correct the misleading information from truncated DR5.

In addition we explored FLT-PET as a more general molecular imaging marker of cellular proliferation to predict therapeutic response at the beginning of anticancer treatment, especially when combining with antiproliferation therapeutics such as Sorafenib. We found that FLT-PET is a sensitive predictor of the response of tumors to therapy at 72 hours of treatment. Differential early changes in FLT-PET uptake predicted the superiority of TRAIL/Sorafenib regimen over cisplatin/docetaxel in CALU-6 tumor-bearing xenograft mice. Taken together, early changes in tumor uptake of FLT-PET are not only predictive of tumor sensitivity to a chemotherapeutic regimen, but also to subtle differences in tumor responsiveness either due to ineffective therapy or superiority of one effective treatment regimen over another. Optical imaging with AngioSense demonstrated decreased vascularity of tumors associated with Sorafenib therapy.

Some minor contributions of this work include strategies to successfully produce and label recombinant soluble TRAIL and Annexin V. TRAIL imaging is safe in preclinical

treatment trial and is more powerful to induce apoptosis than DR4/5 agonistic monoclonal antibodies HGS-ETR1/2. Sorafenib synergizes with TRAIL to induce more prominent apoptosis than single agent therapy and cisplatin/docetaxel in non-small cell lung cancer Calu-6.

In summary multimodality molecular imaging will greatly aid in accelerating targeted anticancer drug approval process and improving survival and response rates in hard-totreat cancers.

6.2 Future work

Imaging cancer DR4/5 expression with near infrared fluorescent dye labeled TRAIL and HGS-ETR1/2 was able to assess cancer cell surface DR4/5 expression level accurately but cannot predict resistance caused by impaired downstream signaling. Complementary imaging of downstream apoptotic markers such as active caspase-3 and phosphatidylserine (imaging with IRDye 800 CW labeled Annexin V shown in this work) can offset mutant DR4/5 misleading information. Future work on this project would follow the directions listed below.

1. Checking other tumor xenografts with a broad range of receptor expression. The current study demonstrated the feasibility of predicting anticancer therapeutic response with death receptor imaging and FLT uptake changes in a few sensitive and resistant tumor xenograft models. Further evaluation in larger study populations with a broad range of receptor expression is warranted.

2. Checking the impact of decoys by using the HGS-ETR1/2. TRAIL imaging shows the expression level of both proapoptotic receptors and antiapoptotic receptors. HGS-ETR1/2

imaging shows only proapoptotic receptors. The impact of decoy receptors on TRAIL sensitivity could be assessed by comparing TRAIL imaging with HGS-ETR1/2 imaging. 3. Combining the death receptor imaging with FLT-PET imaging to predict response prospectively. Basing on normalized death receptor imaging intensity and FLT *s*tandardized *u*ptake value (SUV) change, a TRAIL response index can be derived for prediction of response to TRAIL induced apoptosis.

4. Improving death receptor imaging sensitivity. In the current study, TRAIL treatment dosage in cell culture is nM but in-cell Western assay is mM. To improve sensitivity, we can improve detection sensitivity (*in vitro* from plate scan to slide scan; *in vivo* from animal imager to macroscope, e.g. Nuance) and optimize binding ability (adjust Dye/Protein).

5. In the long run, isotopic labeling of TRAIL and HGS-ETR1/2 may be possible for clinical trial. Tracer dosage of TRAIL and HGS-ETR1/2 will be sufficient for PET imaging. The decay period of 18 F is short (less than 24 hours) so it is feasible to do two PETs to predict the TRAIL treatment response.

6. Early changes in Calu-6 tumor uptake of ¹⁸F-FLT are not only predictive of tumor sensitivity to a chemotherapeutic regimen, but also to subtle differences in tumor responsiveness either due to ineffective therapy or superiority of one effective treatment regimen over another. Future work of this project would be further characterization of the relationship between therapeutic drug half-life and time of measurement of tumor accumulation of ¹⁸F-FLT.

7. The principal assumptions for the use of ¹⁸F-FLT uptake as a surrogate of proliferation are that the tumor utilizes thymidine salvage to a significant degree and that the therapy

induces cell cycle regulation of TK1. If the tumor relies predominately or completely on *de novo* nucleoside synthesis or has defective p53 response (common in tumor cells), ¹⁸F-FLT uptake won't report on proliferation status correctly. In the future, to extend FLT-PET results from Calu-6 to other cell lines, validating these two assumptions is essential. 8. In future cell membrane fluorescence labeling can be added to current work. Preliminary results are presented in Figure 6.1. The CellVue® Maroon is a lipophilic membrane intercalating fluorescent dye with long aliphatic tails (Horan and Slezak 1989). We can label MSTO-211H mesothelioma cell membrane with CellVue® Maroon and inject labeled cells to nu/nu mice subcutaneously or intraperitoneally. After treated with Sorafenib at 64 mg/kg daily for 3 weeks, mice were imaged with the Maestro imaging system. A significant response of mesothelioma to Sorafenib therapy is shown in Figure 6.1 – the treated mouse had markedly less gross tumor burden and marked cell death on histology. CellVue Maroon can identify both subcutaneous and intraperitoneal xenograft tumor cell mass. (Figure 6.1 E and F)

9. Molecular imaging of protein kinases, important regulators of signal-transduction pathways and related to more than 400 human diseases, will greatly facilitate targeted anticancer drug development. (Zhang, Lee et al. 2007; Zhang, Bhojani et al. 2008; Chan, Paulmurugan et al. 2009) We planned to develop an ERK1/2 reporter basing on conformation dependent complementation of firefly luciferase to test inhibition of ERK phosphorylation *in vivo* for our ongoing studies with Sorafenib and TRAIL receptor targeting agents. Dr. Alnawaz Rehemtulla from the University of Michigan kindly provided plasmids, ERK(wt) and MBP(wt), and cell lines, BxPC3-ERK(wt) and BxPC3-MBP(wt), and the domain structure and action mechanism diagram of the

Bioluminescence ERK reporters (Figure 6.2 A and B). We treated BxPC3-MBP(wt) cells with TRAIL and Sorafenib and imaged with IVIS system. Figure 6.2 D suggests that Sorafenib inhibited ERK1/2 activity at the concentration of 64 μ M.

10. Recently the O-glycosylation of TRAIL proapoptotic receptors was discovered to facilitate receptor clustering and caspase-8 activation in tumor cells. Comparing amino acid sequence of DR4/5 predicted conserved extracellular O-glycosylation sites. O-linked glycans regulate functional properties of DR4/5 including their conformation, trafficking and turnover. *In vitro* sensitivity to PARAs is strongly predicted by the expression of the O-glycosylation initiator enzyme N-acetylgalactosaminyltransferase-14 (GALNT14) in *n*on–small cell *l*ung cancer (NSCLC) and other cancer cell lines, and of the *fu*cosyltransferase (FUT) enzymes, FUT3 and FUT6 in colorectal cancer (CRC) cell lines (Wagner, Punnoose et al. 2007). *Immunoh*istochemistry (IHC) assays were developed to measure GALNT14 and FUT3/6 levels in formalin-fixed, paraffin-embedded human NSCLC and CRC tissue (Stern, Padilla et al. 2010). GALNT14 and FUT3/6 could be potential molecular imaging markers for non-invasively predicting response or resistance to PARA treatment *in vivo*.



Figure 6.1 Pilot evaluation of human MPM cell lines in a xenograft mouse models suggests Sorafenib efficacy. *nu/nu* mice were injected IP with human malignant pleural mesothelioma cell line, MSTO-211H cell surface labeled with a fluorescent label, CellVue Maroon. Images above demonstrate (A) untreated (3 weeks daily IP vehicle) (B)

treated with Sorafenib 60 mg/kg daily for 3 weeks (C) exposed untreated tumor with peritoneum resected (D) exposed treated tumor with peritoneum resected (E) large soft tissue IP tumors (F) and smaller tumor lesions demonstrate fluorescence in the expected excitation/absorption spectrum for CellVue Maroon cell surface labeled MSTO-211H utilized for these IP injected tumors. Immediately after sacrifice, xenografted tumor tissues were dissected, fixed in 4% formalin, paraffin-embedded, sectioned in 6 μ m slices and stained with hematoxylin and eosin. Treated tumor nodules demonstrated marked hyalinization and reduce tumor viability (H) compared with untreated tumor (G).

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Figure 6.2 Bioluminescence MEK reporters: (A) The domain structure of the Bioluminescence ERK reporters. Two versions of the Bioluminescence ERK reporters were developed: the ERK(wt) molecule, which contains a specific sequence in ERK1/2 that MEK phosphorylates; and the MBP(wt) molecule, which contains the MBP target sequence that is phosphorylated by ERK1/2. (B) The proposed mechanism of action for the Bioluminescence ERK reporters involves MEK-dependent phosphorylation of the ERK/MBP domain, which results in its interaction with the Phospho-peptide binding domain (left). In this form, the reporter has minimal bioluminescence activity. In the

absence of MEK activity, the N-Luc and C-Luc domains reassociate, restoring bioluminescence activity (right). (C) BxPC3 cells expressing MBP(wt) reporter were treated with TRAIL (100 ng/mL), Sorafenib (64 μ M) and Sorafenib (8 μ M) and imaged with IVIS system.

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