Biomarker and Therapeutic Studies of Antibodies and Small Molecules that Target EGFR

by

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The field of targeted cancer therapy has progressed in recent years with the approval of new oncology drugs. Coupled with the benefits that these agents provide, has come an appreciation for challenges that occur when attempting to translate successful experiments from the laboratory into effective clinical trials. One such challenge has been predicting the optimal dose and schedule to take into clinical evaluation, given the possibility that certain targeted therapeutics may exhibit maximal anti-tumour efficacy well below maximum tolerated doses. Recent work with a targeted antibody to the mouse vascular endothelial growth factor receptor-2 demonstrated that detection of increased levels of its endogenous ligand in the plasma, namely VEGF, could address this issue, as maximal increases in VEGF paralleled optimal drug activity. The VEGF result has become recognized as a potential class effect for this family of inhibitors. This thesis summarizes experiments designed to build upon this discovery by investigating whether the utility of ligand measurement might also apply to drugs that inhibit the epidermal growth factor receptor (EGFR), which have also
received recent regulatory approval, and inhibit angiogenesis as one of their mechanisms of action. In addition, we investigated the potential application of EGFR inhibitors to influence other markers of tumour angiogenesis, specifically their effects on levels of circulating endothelial progenitor cells (CEPs). Finally, we evaluated combination treatment of EGFR inhibition with anti-angiogenic scheduling of chemotherapy.

The EGFR ligand TGFα increased in a dose dependent fashion following treatment with cetuximab, and levels in the circulation paralleled anti-tumour activity. This was a host-dependent effect that was not observed with the lower affinity antibody nimotuzumab. Inhibition of host EGFR also reduced plasma CEPs, but at higher doses these drugs increased off target growth factors VEGF and G-CSF, as well as CEPs. In a model of advanced triple negative breast cancer, the combination of nimotuzumab and metronomic cyclophosphamide was efficacious and well tolerated, leading to a potential new treatment strategy for this aggressive disease. Taken together, these studies identify new and useful applications for EGFR-targeted antibodies, and shed further light on their contributions within the field of tumour angiogenesis and antiangiogenic therapy.
Acknowledgements

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<tr>
<td>AR</td>
<td>Amphiregulin</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BID</td>
<td>Twice Daily</td>
</tr>
<tr>
<td>BTC</td>
<td>Betacellulin</td>
</tr>
<tr>
<td>CEP</td>
<td>Circulating Endothelial Progenitor Cell</td>
</tr>
<tr>
<td>CSF-1</td>
<td>Colony Stimulating Factor-1</td>
</tr>
<tr>
<td>DCE-MRI</td>
<td>Dynamic Contrast Enhanced Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>DLT</td>
<td>Dose Limiting Toxicity</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Essential Medium</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>EPR</td>
<td>Epiregulin</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte Colony Stimulating Factor</td>
</tr>
<tr>
<td>GIST</td>
<td>Gastrointestinal Stromal Tumour</td>
</tr>
<tr>
<td>HB-EGF</td>
<td>Heparin Binding Epidermal Growth Factor</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>KDR</td>
<td>Kinase Insert Domain Receptor</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen Activated Protein Kinase</td>
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<tr>
<td>MTD</td>
<td>Maximum Tolerated Dose</td>
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<tr>
<td>NRG</td>
<td>Neuregulin</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<td>--------------------------------------------------</td>
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<tr>
<td>OBD</td>
<td>Optimal Biologic Dose</td>
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<tr>
<td>PDGFRα</td>
<td>Platelet Derived Growth Factor Receptor Alpha</td>
</tr>
<tr>
<td>PDGFRβ</td>
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<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
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<tr>
<td>PI3-K</td>
<td>Phosphoinositol Triphosphate Kinase</td>
</tr>
<tr>
<td>P/GF</td>
<td>Placental Growth Factor</td>
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<td>PO</td>
<td>Per Os (Orally)</td>
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<td>RECIST</td>
<td>Response Evaluation Criteria In Solid Tumours</td>
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<tr>
<td>RTK</td>
<td>Receptor Tyrosine Kinase</td>
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<tr>
<td>RTKI</td>
<td>Receptor Tyrosine Kinase Inhibitor</td>
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<tr>
<td>SCID</td>
<td>Severe Combined Immunodeficient</td>
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<td>SCF</td>
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<td>SDF-1α</td>
<td>Stromal Derived Factor 1 Alpha</td>
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<td>SDS</td>
<td>Sodium Dodecyl Sulphate</td>
</tr>
<tr>
<td>SID</td>
<td>Once Daily</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transduction and Activator of Transcription</td>
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<td>TGFα</td>
<td>Transforming Growth Factor Alpha</td>
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<tr>
<td>TSP-1</td>
<td>Thrombospordin 1</td>
</tr>
<tr>
<td>VDA</td>
<td>Vascular Disrupting Agent</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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Chapter 1

Introduction

1.1 Targeting tumour angiogenesis for cancer treatment

It is now well established that, in order to expand beyond a few millimetres in size, solid tumours recruit their own blood supply, to provide oxygen, nutrients, and the removal of waste products. The notion that this process of tumour angiogenesis can and should be targeted as a treatment strategy in oncology was largely pioneered by Judah Folkman nearly 40 years ago. As research into this concept gained momentum, the discovery of a substantial number of angiogenic growth factors and inhibitors followed, each of which could theoretically be targeted to suppress tumour angiogenesis. Considered among the most potent of the pro-angiogenic growth factors, vascular endothelial growth factor (VEGF) became a major target of drug development, which resulted in many preclinical successes, followed by clinical evaluation of bevacizumab (Avastin®), a humanized monoclonal antibody that neutralizes human VEGF-A. Preliminary clinical trial results with bevacizumab as a single agent, as well as combination with a standard chemotherapy regimen (capecitabine) for the treatment of metastatic breast cancer were disappointing, raising doubts about the clinical utility of antiangiogenic therapy. This reinforced the feeling that once again clinical results were not able to recapitulate successful preclinical research conducted in animal models; a reality that had
been experienced all too often with other targeted cancer treatment approaches, such as inhibition of matrix metalloproteinases (MMP)\textsuperscript{10-12}.

Despite these notable setbacks, the vision of Dr. Folkman and others for a cancer treatment focused on targeting tumour blood vessels did become a clinical reality, beginning with the regulatory approval of bevacizumab in 2004. Initial Food and Drug Administration (FDA) approval was based on significant prolongations of progression-free and overall survival when bevacizumab was administered in combination with 5-fluoruracil-based chemotherapy in a first-line phase III clinical trial in metastatic colorectal cancer patients\textsuperscript{13}. At this point the future of VEGF-targeting, and the field of angiogenesis as a whole, appeared brighter. Other agents and other strategies to inhibit VEGF-based angiogenic growth signalling were also finding regulatory approval, namely sunitinib (Sutent\textsuperscript{®}) for renal cell carcinoma as well as gastrointestinal stromal tumours (GIST)\textsuperscript{14;15}, and sorafenib (Nexavar\textsuperscript{®}) for renal cell carcinoma as well as hepatocellular carcinoma, in the years that followed\textsuperscript{16}. Even though these latter agents are multitargeted tyrosine kinase inhibitors that bind to more than just VEGF receptors, including VEGFR-2, they induce anti-angiogenic activity, which probably makes a major contribution to their anti-tumour effects; currently there are dozens of such agents in varying stages of clinical trial evaluation for a wide variety of cancers\textsuperscript{16}. 
1.2 Targeting receptor tyrosine kinases

VEGF is a growth factor that initiates signalling through binding to VEGF receptors, which are members of a very large family of receptor tyrosine kinases (RTKs) \(^{17}\). These RTKs are primarily cell surface located, plasma membrane spanning proteins, which are grouped according to structural similarities. Each contains an extracellular domain that permits engagement by the relevant growth factor(s), and a catalytic domain within the intracellular region, which propagates the signal due to phosphorylation events that occur on tyrosine residues (Figure 1). Due to the overexpression, mutation, and/or defective downregulation of a variety of RTKs and/or their corresponding growth factor ligands in a large number of cancers \(^{18,19}\), there has been a rapid expansion in development of targeted agents directed at these pathways. This enthusiasm was undoubtedly initiated prior to the approval of bevacizumab with the successful clinical application of trastuzumab (Herceptin®) for breast cancer \(^{20,21}\) and imatinib mesylate (Gleevec®) for chronic myelogenous leukemia \(^{22,23}\) and GIST \(^{24}\). Both of these agents were designed to inhibit cellular signalling that occurs by tyrosine kinases, and each represents an example of the two important clinical strategies employed for this type of drug development – monoclonal antibodies and small molecule RTKIs.

1.2.1 Monoclonal antibodies vs. RTKIs

There are fundamental differences between these two types of drugs, which are likely to have implications for treatment strategies, side effects and
Figure 1 Receptor tyrosine kinases. A large family of transmembrane receptors containing an extracellular ligand binding domain and catalytic kinase domains, permitting phosphorylation events on tyrosine residues. Receptor families are classified according to structural characteristics. For example, VEGF receptors contain a split kinase domain.
tumour biology. First, and already mentioned above, is the large difference in specificity that is seen. Monoclonal antibodies, whether they are utilized as drugs, laboratory reagents, or to stimulate normal immunologic processes in the body, are highly specific to defined epitopes. They are large protein molecules that will bind to the cell surface, but have limited ability for intracellular penetration. They are administered, generally speaking, via the intravenous route and remain in the circulation for prolonged periods of time, usually days to weeks. These characteristics are in sharp contrast to those of the RTKIs. Most RTKIs are adenosine triphosphate (ATP)-mimetics, as they are designed to structurally compete for occupation of the ATP-binding pocket within catalytic sites in the cytoplasmic portion of the receptor. It is within these sites that the phosphate donation from ATP occurs to facilitate phosphorylation of the tyrosine residues. Because each RTK family member possesses this similar mechanism for signal propagation, most RTKIs will bind to a variety of different receptors, albeit with varying affinity. As smaller and often lipophilic molecules, these drugs gain easy entry to the cell, but many (in the first generation of inhibitors) also rapidly dissociate from their targets and are quickly eliminated, usually within a matter of hours. As such they are administered orally on a frequent, often daily or even twice daily basis. There are other strategies employed to target RTKs, including, but not limited to soluble receptors, immunotoxins, and antisense oligonucleotides. However, the monoclonal antibodies and small molecule RTKIs represent strategies that have proceeded
furthest into clinical use, and their fundamental differences have contributed to interpretation of results in this thesis.

1.3 Challenges in translation of targeted therapy

The difficulties in successful clinical application of promising targeted experimental therapeutics can not be overstated. It is not just the disappointment that follows negative results of clinical trial, but also the tremendous time and financial cost associated with the large phase III clinical trials that must be performed to obtain regulatory approval. Insightful translational research, leading to optimal application of these drugs in patients is therefore vitally important.28.

A key strategy associated with the potential for improved clinical outcomes in patients treated with targeted drugs is the application of appropriate biomarkers.29 Use of biomarkers can be grouped into 3 strategic areas: 1) identification of the appropriate patient population, 2) monitoring treatment response, and 3) pharmacodynamic optimization of drug dose and schedule. Accomplishing these tasks is a requirement for the translation of any oncologic drug, and there are well described protocols to evaluate each of them. Preclinical research models and especially results of phase I trials help to define appropriate tumour types, while ancillary technologies seek to define subsets of the population that may or may not be expected to respond to the drug in question. Well defined parameters, such as the response evaluation criteria in solid tumours (RECIST), are used to define drug activity through objective measurement of tumour response. Finally, pharmacokinetic information and
dose escalation designs in phase I trials aid in determination of the appropriate drug dose and schedule, with the traditional goal in medical oncology of delivering the maximum tolerated dose (MTD) when conventional cytotoxic chemotherapy agents are used.

Despite this well-constructed framework, it is in the translation of newer, targeted drugs, where these standard strategies appear to sometimes break down. A relevant example is the measurement of tumour response. Many successful targeted agents, including those that inhibit angiogenesis (sorafenib is an example), do not necessarily result in dramatic tumour shrinkage. It is in the recent realization of the potential utility of sustained stable disease as a valid endpoint that investigators have sought alternatives to complement RECIST and current clinical trial designs \(^3\)\(^0\)-\(^3\)\(^2\), such as functional imaging with dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) \(^3\)\(^3\)-\(^3\)\(^5\) or positron emission tomography (PET) \(^3\)\(^6\)-\(^3\)\(^9\) to evaluate a drug’s biological effect on the tumour. Finally, in the context of optimal drug dosing, and certainly the most relevant for this thesis, many targeted drugs do not necessarily have a definable MTD, or such a dose is disconnected and perhaps far above that which leads to optimal biologic activity. The result is a need to investigate biomarkers which can define the so-called optimal biologic dose (OBD) \(^4\)\(^0\). Research into this type of biomarker for drugs that target EGFR forms a large part of this thesis.

Even though EGFR inhibitors possess anti-angiogenic properties, as will be discussed below, it is from the latest research into traditional anti-angiogenic inhibitors of VEGF receptor-2, as well as the anti-angiogenic aspects of
traditional chemotherapeutic agents, that this focus on EGFR emerged. Three areas of research took priority for the laboratory with regard to these anti-angiogenic strategies during my graduate program: 1) evaluation of circulating growth factor ligands to guide the use of VEGF receptor inhibitors, in part to determine the drug’s OBD, 2) the contribution of circulating endothelial progenitor cells (CEPs) to angiogenesis, and 3) combining targeted ant-angiogenic agents with low dose, anti-angiogenic scheduling of chemotherapy, more commonly known as metronomic chemotherapy. The goal of this thesis was to investigate these areas with respect to targeting EGFR, a strategy that also became a clinical reality during the same recent time frame as the VEGF-targeted agents mentioned earlier.

1.4 Circulating ligand as a pharmacodynamic biomarker

One major difficulty in clinical trial design for some targeted agents is the potential paucity of dose-limiting toxicities, resulting in a MTD that may be difficult to determine from traditional phase I trials. This presents a challenge in selecting an appropriate dose and schedule to take forward into phase II trial evaluation, the results of which would determine whether a drug is suitable for the considerable investment in a much larger, randomized phase III trial, or should be removed from clinical development altogether – a fate that befalls the majority of experimental therapeutics in oncology. A review of clinical trials with bevacizumab reveals a variety of doses and schedules that have been utilized in phase II and phase III clinical trials, including 5, 10, and 15 mg/kg doses, given
biweekly, or every 3 weeks. However, there may be an MTD for this drug, in the range of 20 mg/kg, defined by severe migraines. So this means that the drug is often administered well below the MTD, e.g. at 5 mg/kg in some cases, as in colorectal cancer patients. It is also clear that the spectrum of side effects seen with bevacizumab and other VEGF pathway antagonists differs from that which is seen with conventional cytotoxic chemotherapy – side effects such as hypertension, proteinuria, epistaxis and/or arterial thrombosis, instead of myelosuppression and/or gastrointestinal disturbance (although these effects can be seen with certain RTKI-type anti-angiogenic drugs, such as sunitinib).

In 2004, Bocci et al in the Kerbel lab described an observation that directly addressed this issue of empirical dose determination, in the context of VEGF signalling inhibitors. They found, using a monoclonal antibody that specifically targets murine VEGFR-2 (flk1) called DC101, a dose-dependent increase in mouse plasma VEGF levels when this antibody was given to mice via the intraperitoneal (IP) route, even after a single dose. This rise in circulating VEGF reached a plateau at higher antibody doses. The starting dose was 50-100 \( \mu g \)/mouse and was extended to 2000 \( \mu g \)/mouse. When compared to preclinical efficacy experiments with this antibody it was determined that the dose range resulting in the highest VEGF plasma levels reached a plateau at approximately 1000 \( \mu g \). Increasing the dose beyond 1000 \( \mu g \) does not result in greater antitumour activity. This result suggested a method to determine rapidly the OBD for this neutralizing antibody (i.e. around 1000 \( \mu g \)) that complimented conventional long-term empirical dose escalation experiments. It is noteworthy that VEGF
increases were observed in normal, non-tumour bearing mice, as the target for DC101 is restricted to murine blood vessels. When certain human tumour xenografts were generated in immunosuppressed mice, such as the high VEGF-expressing MDA-MB-231 breast carcinoma, increased plasma human VEGF was also observed. Human VEGF binds to murine endothelial VEGFR-2, and is considered a very important growth factor source for blood vessels supplying the tumour in this xenograft system. In addition to DC101, other VEGF receptor inhibitors were also tested, revealing a similar finding with a different VEGFR-2 antibody called RAFL-1, but VEGF increases were not observed to a similar extent when VEGFR-1 was targeted with an antibody called MF-1, and no increases were seen with the weakly potent small molecule RTKIs vatalanib (PTK787) and semaxanib (SU5416). However, interestingly, when these and other, more potent VEGF RTKIs were examined in subsequent studies, namely sunitinib, among others, it was revealed that these agents did produce increases in circulating VEGF, and furthermore, this phenomenon has come to be recognized as a class-effect for these agents, along with concomitant decreases in circulating soluble VEGFR-2, both preclinically and clinically\textsuperscript{14,45-47}.

Experiments to address the potential mechanism(s) responsible for these VEGF elevations, at least those induced by an antibody, were reported by Bocci et al\textsuperscript{44}. When mRNA analysis was performed on mouse tissues harvested after 2 weeks of treatment (4 total doses) with DC101, skin, spleen, bone marrow, and heart all showed an increase in VEGF mRNA. The rapid increase in circulating VEGF in these mice, i.e. within a few hours, suggests that pre-formed VEGF
protein likely also plays a role. In addition, Bocci et al also examined platelet release of VEGF in vitro, as the alpha granules of platelets have been reported to store and release VEGF, and platelets themselves express VEGFR-2. When platelet rich and platelet poor plasma was incubated with DC101 no difference in the VEGF content of the supernatant was observed. This result remained consistent when platelet aggregation was stimulated by thrombin.

Building on the work of Bocci et al, we conducted experiments examining other potential mechanisms for the increased circulating VEGF observed with DC101 treatment. We also evaluated other VEGF and VEGFR inhibitors both in vitro and in vivo. The results of these preliminary studies are incorporated into the following paragraphs of this introduction and the figures represent original data from unpublished experiments of AJ Mutsaers and RS Kerbel. These experiments were conducted to gather further information in the VEGF system prior to undertaking studies with inhibitors of EGFR.

We first examined the potential involvement of the endogenous angiogenesis inhibitor thrombospondin-1 (TSP-1), which can bind VEGF. We considered whether this binding of VEGF by circulating TSP-1 could perhaps be released upon drug treatment. When TSP-1 knockout mice were treated with the OBD of DC101 there was no significant difference in the resulting plasma VEGF elevation over that produced in the parental C57bl/6 strain, nor were the basal levels of circulating VEGF significantly different between these groups of mice (Figure 2). We concluded from this experiment that the binding of VEGF by
TSP-1 is unlikely to play a major role in the VEGF elevations observed with DC101 treatment.

Secondly, we addressed the hypothesis of delayed clearance of VEGF by its receptors, since growth factors for many receptor tyrosine kinases are internalized and subsequently degraded after binding to the receptor. A consequence of this was reported by Dai et al \(^5^0\), with colony stimulating factor-1 (CSF-1) receptor knockout mice, which have up to a 20-fold increase in circulating CSF-1, as receptor-bound cellular internalization of CSF-1 represents a major clearance mechanism for this growth factor. VEGFR-2 levels have been shown to decrease in response to VEGF \(^5^1\). In addition, investigators in the field of therapeutic angiogenesis have investigated utilizing VEGF for its potent pro-angiogenic properties, and there is information that documents the circulation time of infused VEGF. Gabrilovich et al showed near complete clearance of 10 \(\mu\)g of \(^{125}\)I-VEGF from BalbC nude mice by approximately 2 hours post treatment \(^5^2\). We tested intraperitoneal (IP) treatment of normal BalbC mice with recombinant human VEGF and also noticed a rapid clearance rate (Figure 3). When these mice were either pre-treated, simultaneously treated, or treated 2 hours post human VEGF injection with DC101 a delayed clearance of rhVEGF was observed (Figure 3). This result does not confirm that the elevated VEGF is due to cellular internalization per se, but it does implicate the consequences of host VEGFR-2 blockade in delaying VEGF clearance. It is possible that other clearance mechanisms besides VEGFR-2 mediated internalization may be altered by DC101, such as the possibility of impaired renal clearance of VEGF
due to DC101 impairment of VEGFR-2 in the glomerulus, where it has been described as having a role in the maintenance of ultrafiltration.53,54

**Figure 2** Treatment of Thrombospondin-1 knockout mice or wild type C57b/6 mice with DC101, the anti-mouse VEGFR-2/flk-1 monoclonal antibody. Mice were treated with a single 800 μg dose IP. A blood sample obtained via cardiac puncture 24 hours after treatment, immediately prior to sacrifice. Mouse heparanized plasma was assayed for mouse VEGF by ELISA (R&D Systems Inc.) p=0.13 by t-test between DC101-treated groups, p=0.69 between control groups.
Figure 3 Human recombinant VEGF injection time trial. 10 μg rhVEGF was injected IP into normal 12 week old female BalbC mice and blood samples were obtained at the time points indicated in A. Human VEGF was assayed from plasma samples using ELISA. Human VEGF levels remained elevated in DC101-treated mice compared to control at 2 and 4 hours after injection in B. DC101 was given 2 hours before, simultaneously, or 2 hours after rhVEGF. 3 mice per group.
1.4.1 VEGF elevations with human drugs – developing an *in vitro* model

The results of Bocci et al with DC101 and similar murine VEGFR-2 targeted monoclonal antibodies were informative, but it was not known whether these findings would be relevant to human antibody drugs that were soon to be entering human clinical trials, because of the species-specific nature of the drug target in mouse studies. It was demonstrated subsequently by Bocci et al that measurement of circulating VEGF in response to growth factor binding, specifically by bevacizumab, could be undertaken in patients, if plasma samples were immuno-depleted of drug-bound VEGF that interferes with ELISA measurement \(^{55}\). We subsequently found plasma VEGF elevations in normal mice after 1 week of treatment with an antibody that binds murine VEGF with very high affinity (Figure 4). It is likely that the ELISA in this instance detected VEGF that was bound to the antibody drug, as well as free VEGF.

In addition to drugs such as bevacizumab that bind VEGF directly, there is interest in drugs which neutralize the ligand-occupying portion of VEGFR-2, as was accomplished by the murine-directed antibody DC101. At the time this thesis study began, there were at least a few human VEGFR-2 neutralizing antibodies, and antibody-like drugs, that were at the crossroads between preclinical testing and early trial evaluation. One of our earliest priorities was to investigate whether there was proof that the circulating VEGF elevations described for DC101 would be relevant to drugs that target the human receptor. We had access to two such drugs: ramucirumab (IMC-1121b), a humanized high affinity phage-display derived monoclonal antibody \(^{56,57}\), and CT322, an
Mouse VEGF in G6.31 dose response
(3 doses given q3d)

Figure 4 Circulating VEGF with mAb against mouse VEGF (G6.31). Antibody was administered to BalbC mice IP at the doses indicated, every 3 days for a total of 3 doses. Blood samples were obtained by cardiac puncture 24 hours after the final dose and mouse VEGF was assayed from heparinized plasma by ELISA.
“adnectin” that contains a VEGFR-2-binding antibody portion fused to the tenth human fibronectin type III domain backbone. Unlike ramucirumab, CT322 also binds murine VEGFR-2, permitting confirmation of results obtained with DC101. Indeed, when administered to normal, non-tumour bearing BalbC mice, dose-dependent increases in plasma VEGF were observed within 24 hours after the first dose was administered IP, as well as after the third dose, when the drug administration was repeated every 3 days (Figure 5). These VEGF increases were observed at dosages that were active and optimal in preclinical cancer models.

Evaluating these drugs for potential effects to cause changes in plasma levels of VEGF ligand when used in humans presented a different challenge. This is especially true with human VEGFR-2 as opposed to other RTKs such as EGFR, as VEGF receptors are not normally found on human tumour cells, and are therefore considered absent in most human tumour xenograft models. Nonetheless, there is a small but increasing body of literature suggesting that VEGFR-2 expression can and does occur in certain human tumours including leukemias, gliomas, melanoma, and prostate carcinoma, at varying stages of progression. A possible implication of these studies is that anti-angiogenic agents may derive at least a portion of their activity from disruption of a potential autocrine growth loop in these tumours, in addition to antiangiogenic effects on blood vessels. We sought to capitalize on this premise by attempting to identify solid tumours with human VEGFR-2 expression. Studies from within the Kerbel laboratory and elsewhere, have demonstrated VEGFR-2 expression...
Figure 5  Treatment of BalbC mice with the VEGFR-2 adnectin CT322. The adnectin was administered to BalbC mice IP at the doses indicated for 1 dose, or 3 doses given every 3 days. Blood samples were obtained 24 hours after the final dose and assayed for mouse VEGF by ELISA. Results from similar experiments have been incorporated into the publication by Mamluk et al, Mabs 2010;2(2).
within the human metastatic prostate carcinoma cell line PC3 (unpublished data, see Appendix 1) as well as melanoma cell lines WM239 and WM9. The PC3 cell line normally secretes small amounts of VEGF compared to many other tumour cell lines grown in monolayer culture (Appendix 1)\(^6\), allowing for easier determination of increases after drug exposure.

After confirming VEGFR-2 expression in the PC3 cell line, an in vitro cell culture system was utilized to quantify VEGF in conditioned media by enzyme linked immunosorbent assay (ELISA) upon incubation of the cells with the ramucirumab antibody for 24 hours. The result was a rapid increase in VEGF in conditioned media (Figure 6), and similar dose-dependent VEGF increases were demonstrated with the adnectin CT322 (Appendix 1)\(^6\). To investigate whether this treatment was affecting cell proliferation or viability, an MTS assay and cell death ELISA were performed. Despite the potential for a VEGF/VEGFR-2 autocrine loop in this system, blockade with 1121b decreased proliferation/viability mildly at a high dose, and increased cell death, but did not alter these parameters to a significant level (Figure 6). We also did not notice a difference in viable cell count using trypan blue staining in these experiments, and the resulting VEGF changes were similar independent of controlling for cell count (Figure 6). Finally, to investigate the possibility that antibody valency, i.e. the capacity for bivalent receptor binding, played a role in the ability of 1121b to increase VEGF in this system, the experiments were conducted with the Fab portion of the 1121b, provided by Imclone Systems, Inc. When cells were incubated with equimolar concentrations of 1121b antibody or Fab fragment, a
Figure 6  Treatment of PC3 with ramucirumab in vitro. Incubation of PC3 human prostate carcinoma cells in monolayer culture with the humanized monoclonal antibody ramucirumab (IMC-1121b) or the Fab fragment for 24 hours. Conditioned media was harvested and assayed for human VEGF by ELISA. Viable cell count was performed with trypan blue stain. MTS and Cell death ELISA performed after 24 hours of drug treatment. Tests performed in triplicate.
similar VEGF increase resulted, independent of valency (Figure 6). Taken together, these in vitro results were encouraging that the circulating VEGF increases described by Bocci for DC101 were at least theoretically possible with drugs such as ramucirumab and CT322. However, these results were obtained exclusively in vitro, so we next set out to determine whether there was a way to investigate these findings in a more relevant biological system. In other words, we wished to test these human drugs in vivo.

1.4.2 VEGF elevations with human drugs – developing an in vivo model

Experiments were performed to recapitulate the in vitro results using a PC3 tumour xenograft model in immunodeficient mice. Unlike the mouse system with DC101, where VEGF and VEGFR-2 can be found from numerous sources throughout the body, the only source of human VEGF and VEGFR-2 in this in vivo model comes from the tumour xenograft itself. Despite the fact that human VEGF is secreted by the tumour, it may not be to a level that is detectable in mouse plasma. Indeed, using PC3, human VEGF was undetectable in mouse plasma when tumours were injected in either subcutaneous locations (Figure 7), or intravenously. We then acted on information that angiogenic cytokines may be better elaborated from intraperitoneal locations \(^69\)\(^70\), and inoculated mice with PC3 IP. Once a relatively high tumour burden was reached the mice were treated with a single dose of 1121b antibody. The result was a dramatic elevation in human VEGF in the peritoneal cavity fluid that had developed (Figure 7). In addition, it appeared from initial experiments that the volume of this ascites
Figure 7 Treatment of PC3 with ramucirumab in vivo. PC3 was implanted IP in SCID mice and grown to a high tumour burden. A single dose of ramucirumab was administered IP and plasma and ascites were sampled 24 hours later (or at the times indicated).
produced was higher in mice that had received treatment with 1121b, possibly due to binding of the human VEGF to unblocked mouse VEGFR-2 in the peritoneal vasculature or elsewhere, increasing vessel permeability and ascites formation. Dose-response and time course studies were also conducted with this model, and revealed VEGF increases at the lowest dose (2 μg) and earliest time point (0.5 hours) evaluated (Figure 7).

These in vivo results were also encouraging, and indeed Imclone systems/Eli Lily and Adnexus/Bristol Myers Squibb have evaluated circulating VEGF in early phase clinical trials of ramucirumab and CT322, respectively. Results of these clinical trials showed rapidly increased levels of circulating VEGF 71;72 (Appendix 2), similar to what we observed in mice. In the case of ramucirumab, no dose response was observed in the phase I trial, but the dose-response range was narrow: 2 mg/kg to 16 mg/kg 71 (Appendix 2). If our results are correct, it could mean that the lowest dose (2 mg/kg) was already above the OBD.

1.5 Induction/elevation of off-target growth factors and circulating endothelial progenitor cells

One interesting development that has unfolded since the publication by Bocci et al regarding the increase in circulating VEGF in normal mice treated with DC101 has been the realization that this finding is not restricted to antibodies. Neither of the small molecule RTKIs evaluated in Bocci's study have proceeded successfully to regulatory approval. However, newer drugs such as sunitinib and
sorafenib are in clinical practice, and as Ebos et al demonstrated in 2007, their administration also leads to VEGF increases, with concomitant decreases in soluble VEGFR-2 in normal mice. Prior to this study, it was thought that much, if not all of the VEGF increase was coming from the tumour. Indeed, recently a study in which sunitinib was administered to healthy human volunteers showed an increase in plasma VEGF and confirmed that it was tumour-independent. It is not certain whether the mechanism responsible for this effect is the same for these RTKI drugs, where ligand engagement of the receptor can still occur, as it is for neutralizing antibodies like DC101 or ramucirumab, or an adnectin like CT-322. There is evidence for differences in mechanisms since the onset of VEGF increase is much more rapid with the use of VEGFR-2 neutralizing antibodies. A second result from the study by Ebos et al is the demonstration of dose dependent increases in a number of other growth factors/cytokines in circulation upon treatment of normal mice with sunitinib. We know that sunitinib inhibits a wide range of RTKs. Some of the cytokines and chemokines that were shown to be elevated bind to receptor targets of the drug such as stem cell factor (SCF) which binds c-kit, and placental growth factor (PIGF) which binds VEGFR-1. However, elevations were also documented for cytokines such as granulocyte colony stimulating factor (G-CSF), stromal derived factor-1α (SDF-1α), and osteopontin, whose receptors are not targets of sunitinib. These factors nevertheless support and/or amplify angiogenesis, through mechanisms that include their influence on bone-marrow derived proangiogenic cells, such as circulating endothelial progenitor cell (CEP) populations. Given that sunitinib
was subsequently shown by Paez-Ribes et al \(^{76}\), as well as Ebos et al \(^{77}\) to accelerate invasion and metastasis in certain mouse tumour models after short term treatment, there is a compelling rationale for characterizing the contributions made by these growth factors to changes in tumour biology and possibly to toxic side effects. The importance of the issue is even more concerning since the drug is approved for treatment on an intermittent schedule, i.e. 4 weeks of treatment followed by a 2-week break \(^{15}\). Understandably, there is concern regarding the biologic consequences of tumour “flare” or “rebound” that has been observed during the break period \(^{78-81}\). In addition to this intermittent schedule, treatment breaks due to toxicity may be very common with such drugs in general oncology practice \(^{82}\). Finally, the increase in plasma levels of multiple proangiogenic factor changes is not restricted to sunitinib, as other RTKIs, such as vandetanib (Zactima®) and pazopanib (Votrient®) have shown similar results, with elevated VEGF, PGF, SDF-1α, and G-CSF \(^{45,83}\), and characterization of circulating levels of proangiogenic factors after treatment with other targeted anti-angiogenic agents, including antibodies, is ongoing.

Independent of the research that has taken place with regard to increases in circulating levels of various growth factors when anti-angiogenic agents are utilized, has been the documentation and characterization of the role of bone marrow-derived cells to the process of angiogenesis. Circulating endothelial progenitor cells (CEPs) are an example of bone-marrow derived cells that may play a role in angiogenesis \(^{84,85}\). The Kerbel laboratory, and many others, have been identifying these cells using 4-colour flow cytometry and the surface
markers CD45\(^{-}\), CD117\(^{+}\), CD13\(^{+}\), VEGFR-2\(^{+}\). Examples of other bone marrow derived cell types that may have a pro-angiogenic role include Tie-2 expressing monocytes (CD45\(^{+}\), Tie-2\(^{+}\), CD11b\(^{+}\)), recruited bone marrow derived circulating cells (CD45\(^{+}\), CXCR4\(^{+}\), VEGFR-1\(^{+}\), VEGFR-2\(^{-}\)), tumour associated stromal cells (CD45\(^{+}\), CD117\(^{+}/\)Sca1\(^{+}\), VEGFR-2\(^{+}\)), and CD11b/Gr1\(^{+}\) myeloid suppressor cells\(^{86}\). The contribution that CEPs and other bone-marrow derived cells make to the process of angiogenesis is controversial\(^{84,87-91}\), and the full complement of cytokines that influence their behaviour is not known, although they include VEGF and SDF-1. There have been two interesting uses documented for CEPs that may be relevant to the translation of targeted anti-angiogenic drugs. The first is the use of CEP suppression as a biomarker of the OBD for certain anti-angiogenic agents or anti-angiogenic scheduling of (metronomic) chemotherapy\(^{92,93}\), and the second is exactly the opposite: the “surge” of these cells that can occur almost immediately after treatment with vascular disrupting agents\(^{94}\) or MTDs of certain cytotoxic chemotherapy agents\(^{74}\).

In 2005 Shaked et al elegantly documented the correlation of endogenous CEP levels with angiogenic potential in mice\(^{92}\). To do so required assessment of CEPs in a variety of mouse strains, including “knock out” and transgenic models, influenced by potent regulators of angiogenic ability, such as VEGF and Thrombospondin-1 (TSP-1). In addition, and perhaps more importantly for the focus of this thesis, the authors were able to show a correlation between CEP levels and the OBD of the targeted anti-angiogenic drugs DC101, as well as ABT-510, a peptide mimetic of thrombospondin-1\(^{92}\). Since that time it has also
been demonstrated that a reduction in CEPs might be a marker for optimal doses of cytotoxic chemotherapy, when delivered in a low dose continuous, anti-angiogenic, or “metronomic” schedule \(^93\). This strategy for inhibiting angiogenesis is described in detail in the next section.

If CEPs make an important contribution to adult angiogenesis (or “vasculogenesis”, the term often used when discussing bone marrow contributions to the process) it may depend upon insult to the vessels inside a solid tumour. In fact, recruitment of CEPs may also be important for the body’s response to other vascular insults as well, such as acute thromboembolic events. The field of therapeutic angiogenesis has therefore been interested in these cells for their role in repair of vascular conditions such as myocardial and cerebral infarction \(^95\). Perhaps the strongest and fastest disruptors of blood vessels are a class of drugs referred to as “vascular disrupting agents” (VDA). In 2006 Shaked et al described the acute recruitment of CEPs to tumour sites in response to VDA treatment \(^94\), and this was followed in 2008 by demonstration that similar homing of these cells from the bone marrow to tumours could occur rapidly following treatment with certain chemotherapy drugs, such as paclitaxel and cyclophosphamide \(^74\). Fortunately, this work also demonstrated that this vasculogenic rebound, which can accelerate tumour repopulation, could be blunted by interfering with the pro-angiogenic stimulus with drug treatment, e.g. using anti-VEGFR2 blocking antibodies \(^74\). The improved efficacy seen with chemotherapy when combined with anti-angiogenic agents could therefore be explained, in part, by a suppression of the CEP mobilization/tumour colonization
response. The study by Shaked et al also confirmed the potential for involvement of one of the off-target cytokines implicated in the sunitinib experiments introduced earlier, namely SDF-1α, as a stimulator of CEP recruitment. The angiogenic and chemosensitization properties of CEPs make these cells attractive for study with respect to other targeted therapies that are anti-angiogenic and used with cytotoxic chemotherapy. Inhibitors of EGFR are a recently approved class of drugs that fits this description.

There is scepticism from some scientists about the biological relevance, and indeed the existence of a bone marrow derived endothelial progenitor cell. Identification and quantification of these rare cells via flow cytometry, as well as histologically, is inherently challenging. There has been great effort expended to standardize protocols for evaluation, however the discordant results between laboratories remains a significant hurdle to the study of these and other rare bone marrow-derived cell populations, and acceptance by the scientific community. The studies in this thesis regarding CEP levels in mice upon treatment with EGFR-targeted drugs have benefitted greatly from the established track record of the Kerbel laboratory for identification and quantification of these cells using four colour flow cytometry.

1.6 Chemosensitization with low dose continuous chemotherapy

There have been a wide variety of compounds/agents/drugs that have been shown to inhibit angiogenesis. Examples of compounds with demonstrated anti-angiogenic activity, that have undergone evaluation in clinical trials include
fumagillin analogues, thalidomide, endostatin, and cox inhibitors. Due to the approval of bevacizumab as the first targeted anti-angiogenic drug, and the subsequent approval of the RTKIs sunitinib, sorafenib, and pazopanib, the field of anti-angiogenic therapy has largely focused on RTK targeting in general, and the VEGF pathway in particular. Perhaps unappreciated as a result of this focus on targeted therapy was the potential contribution that classical cytotoxic chemotherapy agents themselves may play in angiogenesis inhibition. As chemotherapy drugs target rapidly dividing cell populations, they are generally not considered to be as specific for cancer cells as is expected with the newer generation of targeted oncology drugs. However, the endothelial cells within a growing tumour must also be included as rapidly-proliferating cells along with bone marrow progenitor and gastrointestinal epithelial cells. When looked at from an anti-angiogenic perspective, the “liability” that many chemotherapeutic schedules would appear to have is the obligatory break period required following administration of the MTD, which permits the regrowth/repopulation of the damaged tumour endothelium. Such breaks have been postulated to facilitate tumour cell repopulation after exposure to cytotoxic agents or radiation. What about endothelial cells in the tumour vasculature? In 2000, two separate research groups addressed the anti-angiogenic potential of more frequent/continuous dosing of certain chemotherapeutic agents. The resulting inhibition of tumour angiogenesis from these schedules outperformed intermittent scheduling at the MTD. Of course, elimination of the break period necessitated lowering the dose of the drugs that were used. Browder et al were
able to demonstrate endothelial cell-specific apoptosis within the tumour vasculature in response to cyclophosphamide that preceded the same effect in tumour cells, and was maximized with close repetitive (weekly) drug exposure. Notably, experiments were also conducted in cancer cells already made resistant to cyclophosphamide administered in a conventional dose and pulsatile schedule. The study by Klement et al emphasized the importance of combination approaches. Combining chemotherapy that targets the endothelium with an anti-angiogenic drug aimed at the VEGF pathway is well suited to neutralize the pro-survival response of the endothelium afforded by VEGF and thus bring about an overall superior anti-tumour effect. In this study the antiangiogenic drug was DC101, and its combined use represented improvement over metronomic chemotherapy when administered alone. This study stimulated many of the current phase II metronomic chemotherapy clinical trials utilizing combination approaches with a targeted anti-angiogenic drug, such as bevacizumab.

The anti-angiogenic mechanisms involved in metronomic scheduling of chemotherapy are likely numerous, and have been reviewed. They include alteration in the balance between pro-angiogenic growth factors and/or upregulation of endogenous inhibitors of angiogenesis, as well as the potential suppression of bone marrow derived CEPs. Early clinical trials have demonstrated a correlation between favourable clinical outcome with metronomic cyclophosphamide + methotrexate and reduction in circulating VEGF levels. Others have shown an upregulation of the endogenous inhibitor of angiogenesis thrombospondin-1 (TSP-1) with metronomic chemotherapy, which appears
to correlate with patient benefit\textsuperscript{113}. We know that CEPs can be mobilized out of the bone marrow in response to a number of pro-angiogenic factors, and are also considered a potential target of anti-angiogenic treatment strategies\textsuperscript{92}. However, circulating levels of these cells are also impacted by chemotherapy when the drugs are utilized in either an MTD or metronomic fashion\textsuperscript{93,114}. Using a model of human lymphoma, it was shown that CEP levels fall markedly and abruptly when MTD chemotherapy is administered, but only to rapidly rebound during the break period (similar to the rebound of other bone marrow cells). This compensatory rebound in CEPs was not observed with metronomic scheduling of cyclophosphamide, where the drug was administered at lower weekly doses, or continuously through the drinking water\textsuperscript{114}. Indeed, marked suppression of CEP levels was observed\textsuperscript{114}. Thus, continuous application of certain chemotherapy drugs at lower doses appears to suppress the levels and/or effects of these CEPs, while MTD schedules can promote bone marrow cell recruitment, as part of a vessel repair response. If bone marrow derived CEPs make a significant contribution to angiogenesis, including vessel repair during break periods, the suppression (or lack of induction) of these cells by metronomic chemotherapy could result in a significant anti-angiogenic effect, particularly when chemotherapy is partnered with a separate targeted agent\textsuperscript{96}.

Interestingly, the human clinical trial that may have spawned the most interest in further evaluation of this approach in solid tumours did not involve combination therapy with a targeted anti-angiogenic drug. This study, by Colleoni et al in 2002, used daily low dose (50 mg) cyclophosphamide and twice-
weekly methotrexate (10 mg in total) in 64 women with advanced breast cancer. An overall response rate of 32% was observed in this heavily pre-treated population, which included 2 complete responders, 10 partial responders, and 12 patients with stable disease lasting 6 months or longer. In addition to this clinical trial, and others in breast cancer patients where metronomic scheduling of chemotherapy has been combined with relevant targeted agents, such as bevacizumab, trastuzumab, or letrozole, clinical results have been reported in many other cancers including, but not limited to, colorectal carcinoma, ovarian carcinoma, renal cell carcinoma, prostate cancer, non-small cell lung cancer, non-Hodgkin’s lymphoma, multiple myeloma, melanoma, angiosarcoma, soft tissue sarcoma, malignant glioma, among others – this list continues to grow. Given the potential benefits of sustained pairing of metronomic chemotherapy with targeted anti-angiogenic agents, and the ever growing list of potential clinical applications, we sought to examine whether recently approved drugs that target EGFR, which also are thought to have an anti-angiogenic component to their overall mechanism of anti-tumour action, could represent suitable partners for this combination treatment strategy.

**1.7 EGFR as a therapeutic target**

The first non-hormonal targeted agents to be approved for use in oncology included rituximab, trastuzumab, and imatinib. The targets for these agents (CD20, HER2, and bcr/abl, kit, and others) were found preferentially expressed
by the cancer cell population, and by identifying this target in a patient’s tumour, these agents could be effectively applied. Subsequently, there has been clinical approval of a number of targeted agents within another important signalling pathway, namely the EGFR family 135. In fact, EGFR represents one receptor within the larger erbB family, in which HER2 is also a member.

The erbB family consists of four transmembrane receptors, including EGFR (HER1/ErbB-1), HER2 (erbB-2/neu), HER3 (erbB-3) and HER4 (erbB4) 136. EGFR is a 170 kDa RTK protein, which contains an extracellular ligand-binding domain, a transmembrane domain, and an intracellular tyrosine kinase domain (Figure 1). Family members form homodimers and heterodimers with each other, downstream signalling responses are pleiotropic, and perhaps most significant for this thesis, there are (at least) seven genetically distinct ligands that bind EGFR, and in some cases, other family members as well 137. Although an important receptor in the biology of many breast and ovarian cancers, HER2 does not bind ligand, and HER3 is kinase deficient. However, through homo- and heterodimerization, these receptors are still able to participate in signalling processes, impacting mitogenesis, apoptosis, cell motility, differentiation or dedifferentiation, among others.

EGFR ligands include epidermal growth factor (EGF), transforming growth factor-α, (TGFα), amphiregulin (AR), epiroregulin (EPR), heparin-binding EGF (HB-EGF), betacellulin (BTC), and neuregulin G2β (NRG). The binding of ligand triggers receptor dimerization/aggregation and cellular internalization, while also activating the intracellular tyrosine kinase domain, resulting in phosphorylation
events at tyrosine residues, which in turn initiate a cascade of intracellular signalling pathways. Downstream pathways activated by EGFR include components of the ras/mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3-K), signal transducer and activator of transcription (STAT), protein kinase C and phospholipase D. In many cases the signal can be terminated by endocytosis of the ligand-receptor complex.

Notable differences among the ligands that bind EGFR include their affinity and the intracellular trafficking/degradation that occur upon internalization with bound receptor. Unlike EGF, which is targeted for degradation in the endosome, TGFα may dissociate from the receptor in this acidic environment and be recycled to the cell surface. Additionally, AR and EPR bind to the receptor with a lower affinity than TGFα, EGF, or other family members.

EGFR expression occurs in many cells of the body, including both epithelial and mesenchymal lineages. The extent to which EGFR is overexpressed in tumours is controversial, but estimates vary between 30-50%. Variations in methodology and standardization contribute to this variability; but a number of common cancers overexpress this receptor. In addition to overexpression, proliferation of malignant cells may be triggered by receptor mutation, as well as ligand dependent and independent signalling.

Types of drugs used to inhibit EGFR fall into two categories: monoclonal antibodies and RTKIs. Among the antibody drugs, cetuximab (Erbitux®) was approved by the FDA in 2004 for the treatment of EGFR-expressing colorectal cancer that has progressed after irinotecan therapy, or in patients who are
intolerant to irinotecan-based regimens, and later for treatment after failure of oxaliplatin-containing regimens \(^\text{13}\). The drug is also approved for use with irinotecan in patients that are refractory to irinotecan-based chemotherapy. In head and neck cancer cetuximab is approved for use with radiation therapy for the initial treatment of locally or regionally advanced squamous cell carcinoma, or as monotherapy for recurrent or metastatic squamous cell carcinoma in patients that have failed platinum-based therapy. This chimeric antibody was developed through combination of the variable regions of mouse anti-EGFR antibody mAb 225 with human IgG1 constant regions. The purpose of the chimeric approach was to reduce the potential for an anti-mouse immunological reaction when the drug is given to patients. Other monoclonal antibodies that are approved and/or in late stage clinical trial evaluation include panitumumab (Vectibix\(^\circledR\)), which is of the IgG2 type, matuzumab, and nimotuzumab, all of which are fully humanized. Panitumumab is approved for EGFR-expressing colorectal cancer as monotherapy following disease progression on or following fluoropyrimidine, oxaliplatin, or irinotecan-containing chemotherapy regimens. Notably, k-ras mutations in codon 12 or 13 are associated with lack of response in colorectal cancer patients to both panitumumab and cetuximab \(^\text{144}\), and these drugs are only recommended for use in colorectal cancer patients with tumours that have wild type k-ras. Late stage clinical trials are ongoing with each of these agents in a variety of tumour types.

The small molecule EGFR RTK drugs gefitinib (Iressa\(^\circledR\)) and erlotinib (Tarceva\(^\circledR\)) are approved as single agents for treatment of advanced non small
cell lung cancer, and in the case of erlotinib, chemotherapy-naive pancreatic cancer in combination with gemcitabine. Another RTKI, lapatinib (Tykerb®), is a dual inhibitor of EGFR and HER2, and has been approved in combination with capecitabine for the treatment of HER2+ metastatic breast cancer that has been treated with anthracycline and taxane chemotherapy plus trastuzumab. The spectrum of clinical application of these drugs may be forever changing, as newer clinical trials are completed.

1.7.1 Contributions of EGFR to angiogenesis

In addition to the direct actions of EGFR targeted agents on cancer cells, EGFR blockade may inhibit angiogenesis. Much of this work has focused on the relationship between EGFR and VEGF. In 1997 Viloria-Petit et al in the Kerbel laboratory described down-regulation of VEGF in vitro and in vivo upon treatment of EGFR+ tumours with cetuximab 145, and subsequently, the downregulation of other pro-angiogenic factors interleukin-8 (IL-8), and basic fibroblast growth factor (bFGF). Reduction in xenograft blood vessels and inhibition of tumour growth was also described by others 146. Cross-talk between oncogenes, such as EGFR, and promoters of angiogenesis, such as VEGF, has been described in a number of systems, reinforcing the implication that successful targeting of certain oncogene products can have anti-angiogenic consequences which contribute to efficacy 147-149. Examples of other common oncogenes that can promote angiogenesis in this way include HER2/herB2 145, ras 150, and the bcr-abl fusion protein targeted by imatinib 151, among many others.
Another aspect of angiogenesis that has been linked to EGFR targeting has been the expression of this receptor on (tumour-associated) blood vessels. In theory, even if tumour cell populations were negative for EGFR expression, an anti-angiogenic effect could still result from targeting blood vessel expression of the receptor. Indeed, it has been demonstrated that high growth factor production from the tumour itself, specifically TGF\(\alpha\), can upregulate vascular EGFR expression, making it more likely to respond to EGFR inhibition\(^{152-155}\). Contributions to angiogenesis by EGFR are now well documented in the area of tumour vessel EGFR expression, and the influence EGFR signalling has on the potent pro-angiogenic factor VEGF\(^{156}\). It follows that we wish to examine whether EGFR drugs also remain relevant to the more recent applications of translational angiogenesis research.

1.8 Hypotheses and aims

As the field of angiogenesis has evolved to investigate such areas as the pharmacodynamic biomarker potential of growth factor ligands, the contribution of bone marrow derived cells, such as CEPs, and optimal combination treatment approaches with traditional cytotoxic chemotherapy (the way many of these agents found application and approval) administered in metronomic schedules, we sought to investigate the nature of the contribution of recently approved EGFR inhibitors, in particular the monoclonal antibodies cetuximab and nimotuzumab, to these processes.
The hypotheses of this study are:

1. On-target ligand elevations described for the anti-murine VEGFR-2 neutralizing antibody DC101, and demonstrated for the anti-human VEGFR-2 antibody ramucirumab and the adnectin CT322, will apply to neutralizing antibodies for EGFR, such as cetuximab and nimotuzumab, but may not be relevant for EGFR RTKIs that still allow ligand engagement.

2. Off-target growth factor/cytokine/chemokine changes described for potent VEGF RTKIs sunitinib and sorafenib, will occur with EGFR inhibitors, however the spectrum of changes observed may be unique to each drug evaluated.

3. Given the demonstrated influence of EGFR inhibition on suppressing pro-angiogenic growth factors, such as VEGF, and the documented role these factors have on the mobilization of CEPs, EGFR targeted drugs utilized at the OBD will decrease CEPs in mouse peripheral blood.

4. Monoclonal antibodies that inhibit EGFR will enhance metronomic chemotherapy in appropriate preclinical models of advanced colorectal and breast cancers.
Specific Aims:

1. Investigate potential alterations in EGFR ligands from conditioned media in EGFR\(^+\) tumour cell lines grown in monolayer culture upon incubation with EGFR inhibitors; utilize the in vitro system to examine the potential mechanism(s) responsible, and investigate whether optimal ligand changes predict the OBD for tumour treatment in preclinical models both in vitro and with an in vivo ascites model.

2. Characterize on-target ligand and off-target growth factor changes in normal mice treated with an anti-murine EGFR antibody as well as select EGFR RTKIs, the effects of EGFR inhibitors on CEPs in the BalbC mouse strain, and the potential influence of concurrent chemotherapy on ligand/growth factor changes.

3. Measure circulating EGFR ligands in plasma from cancer patients treated with EGFR monoclonal antibodies to investigate clinical proof of principle.

4. Utilize in vivo xenograft models of colorectal carcinoma and triple negative breast carcinoma to evaluate therapeutic benefit (tumour growth delay and/or metastatic suppression) of a monoclonal antibody that inhibits EGFR used with metronomic chemotherapy.
Chapter 2

Cetuximab Causes a Dose-Dependent Increase in Circulating Transforming Growth Factor-α That Acts as a Pharmacodynamic Marker for Optimal Biologic Dosing

2.1 Abstract

The objective of this study was to characterize treatment-induced circulating ligand changes during therapy with EGFR inhibitors and evaluate their potential as surrogate indicators of the optimal biologic dose (OBD). Conditioned medium from human tumor cell lines, ascites fluid from tumor xenografts, and plasma samples from normal mice as well as colorectal cancer patients were assessed for ligand elevations using enzyme-linked immunosorbent assay following treatment with cetuximab (Erbitux®), nimotuzumab, an anti-mouse EGFR neutralizing antibody, or a small molecule EGFR tyrosine kinase inhibitor (RTKI). A rapid elevation in human TGFα was observed in all cell lines after treatment with cetuximab, but not with nimotuzumab or small molecule inhibitors. The elevation showed a dose-response effect and plateau that corresponded to the maximal decrease in A431 proliferation in vitro and HT29 tumor growth in vivo. The TGFα increase was exacerbated by ongoing ligand production and cleavage from the plasma membrane, but did not involve transcriptional up-regulation of TGFα or the matrix metalloproteinase TACE/ADAM17. Elevations in plasma TGFα, amphiregulin, and epieregulin were also detected in normal mice.

*Portions of this chapter have been published by Mutsaers AJ et al, Clin Cancer Res 2009; 15(7):2397-2405.
treated with an anti-mouse EGFR monoclonal antibody, illustrating a host tissue-dependent component of this effect in vivo. Finally, circulating TGFα increased in the plasma of 6 patients with EGFR-negative colorectal tumors during cetuximab treatment. Treatment-induced increases in circulating ligands, particularly TGFα, should be serially assessed in clinical trials of anti-EGFR therapeutic antibodies as potential biomarkers to aid in determination of the OBD.

2.2 Introduction

With increasing knowledge of the molecular mechanisms that govern carcinogenesis and tumour progression has come the discovery and development of numerous new drugs designed to specifically target the relevant molecular pathways involved. The EGFR represents one such target. A number of drugs targeting this receptor have been approved for cancer treatment and others are being evaluated in late phase clinical trials. Cetuximab is a chimeric anti-human EGFR monoclonal antibody that has recently been approved for use in metastatic colorectal carcinoma as well as head and neck squamous cell carcinoma. However, despite the recent success of targeted therapy in certain clinical oncology trials, including those utilizing cetuximab, there is a need for strategies to improve efficacy, as well as reduce toxicity.

One difficulty has been establishing the dosage that will effectively inhibit the intended molecular target in the tumour and/or patient. Many targeted drugs
do not elicit tumour regression and/or potent toxic side effects, such as myelosuppression, characteristic of conventional cytotoxic chemotherapeutics. The successful application of many of the new molecularly targeted anti-cancer drugs requires determination of the OBD, since unlike traditional chemotherapy, the MTD is frequently not used and sometimes cannot be applied because of the absence of dose limiting toxicities (DLT). Even if DLTs and a MTD can be defined, the optimal biologic/therapeutic activity may be seen at a dose below the MTD – the so called OBD. Thus, determining the OBD is, by definition, highly empirical, which increases the odds of selecting suboptimal or inadequate doses for clinical trials, increasing the risk that such trials will show lesser or minimal, if any treatment benefit.

A drug-induced increase in circulating VEGF has emerged as a “class effect” for patients treated with a variety of drugs that target VEGF receptor pathways. This effect, which has been observed clinically with both VEGFR-2 antibodies and small molecule RTKIs, prompted us to examine EGFR-targeted agents to determine whether similar increases in TGFα or VEGF might occur. Given that therapeutic monoclonal antibodies, such as cetuximab, compete for the ligand-binding site on EGFR, the specific objective of this chapter was to determine and characterize changes in circulating EGFR ligands in response to the administration of the monoclonal antibodies cetuximab and nimotuzumab, as well as the anti-mouse EGFR antibody ME1. Small molecule inhibitors of EGFR erlotinib, gefitinib, and vandetanib were also evaluated, as
previous studies with VEGF inhibitors have revealed ligand elevations following treatment with RTKIs as well as antibodies.

2.3 Methods

2.3.1 Tumour Cell Lines and Culture Conditions

PC3 prostate, DU145 prostate, MDA-MB-231 breast, BxPC3 pancreatic, HT29 human colorectal and A431 epidermoid carcinoma cell lines were obtained from American Tissue Culture Collection (Rockville, MD). The GEO colon carcinoma cell line was a gift from Dr. Lee Ellis. The SW839 and Caki-1 cell line confirmatory experiments were performed by scientists at Imclone Systems (New York, NY). All tumour cell lines were maintained in tissue culture in DMEM supplemented with 5% FBS, except HT29 was grown in RPMI 1640 supplemented with 5% FBS. All cell lines were grown in monolayer culture to 75-90% confluence, detached, made into single cell suspensions using 0.05% trypsin-0.03%EDTA (Invitrogen) and washed once with complete medium for in vitro experiments, or followed by two washes in FBS-free medium prior to injection in vivo. Cells were counted using Trypan Blue and adjusted to the desired concentration for plating or injection.

2.3.2 Reagents and Drugs

Cetuximab, and an anti-mouse specific EGFR neutralizing monoclonal antibody (ME1) were provided by Imclone Systems Inc. (New York, NY). Nimotuzumab was provided by YM Biosciences Inc. (Mississauga, ON). These
antibodies were diluted in media for in vitro experiments, and 0.9% sterile normal saline before intraperitoneal injection in vivo. The activity of the anti-murine EGFR neutralizing antibody has been described previously \(^{172}\). Erlotinib (Tarceva®) was generously provided by Genentech (San Francisco, CA). Vandetanib (Zactima®) was generously provided by Alan Barge at AstraZeneca (Macclesfield, UK). Gefitinib (Iressa®) was a gift from Dr. Ian Tannock. Each RTKI was diluted in DMSO for use in vitro \(^{173}\). Final DMSO concentration was \(<1\%\) and vehicle controls were included. Erlotinib and vandetanib were given by p.o. gavage of homogeneous suspension in 1% Tween 80 in saline. Cycloheximide (Sigma, St Louis, MO) and TAPI-1 (Peptides International Inc., Louisville, KY) were also reconstituted in DMSO for use in vitro.

### 2.3.3 Assessment of surface EGFR expression

Cells were prepared as described above and examined by flow cytometry using a BD Caliber and PE-conjugated anti-human EGFR primary antibody and isotype control from BD Pharmingen. One million cells were placed in each of 3 tubes (for unstained, isotype control, and EGFR stained cells, respectively). Cells were centrifuged at 16000 g at 4 °C for 5 minutes, then resuspended in Hank's balanced salt solution in 2% FCS (HBSS-2%-FCS). 20 μl of antibody (or HBSS-2%-FCS) was added to each tube and incubated for 30 minutes on ice, with gentle agitation twice during the incubation period. Tubes were washed twice with HBSS-2%-FCS, then 0.6 ml of HBSS-2%-FCS containing 1 μg/ml 7AAD was added. Samples were filtered through a 5 ml filter top Falcon tube
and stored protected from light until analysis. Results were displayed by mean fluorescence intensity

2.3.4 In Vitro Dose-Response and Time Course Experiments

Cells were plated in 24-well plates at 1x10^5 cells/well and incubated until they reached 75-90% confluence. Fresh medium containing drug at the specified concentrations was then added to each well in triplicate. Wells containing drug and media, but not cells, were also included to assess potential cross reactivity with the ELISA. Cells were incubated in the presence of drug or vehicle for 0.25-48 hours. Conditioned medium was centrifuged at 16000g for 5 minutes and supernatant was frozen at -80 °C until assayed. For ligand determination in lysates, medium was removed and cells were rinsed with cold PBS, scraped from plates in cold lysis buffer (20 mM Tris pH 7.5, 137 mM NaCl, 100 mM NaF, 10% Glycerol, 1% NP40, 1 mM Na2VO4), centrifuged and resuspended in cold lysis buffer. Protein content of lysates was quantified using Bradford reagent (BioRad, Hercules, CA) and standardized using known amounts of bovine serum albumin (Gibco, New York, NY). For the Bradford assay, 800 μl of standard and sample were pipetted into a clean, dry test tube and 200 μl of dye reagent was added and each tube was vortexed. Tubes were incubated at room temperature for 5 minutes (minimum) and absorbance was measured at 595nm.
2.3.5 Growth factor ELISA Analysis

Human TGFα, AR, EGF, HB-EGF, BTC, and VEGF were assessed using commercially available sandwich ELISA assays or DuoSets (R&D Systems, Inc.) following manufacturers instructions, with the following exceptions: In experiments with low signal detection, sample was added with less or no dilution to the wells in order to maximize the signal. All other samples were diluted as per manufacturer’s instructions. Human EPR was assessed by ELISA, according to the method of Khambata-Ford et al 174, using antibodies obtained from R&D Systems, Inc. Optical density was determined using the microplate reader Benchmark Plus (Bio-Rad Laboratories, Canada) set to 450 nm with a wavelength correction set to 540 nm. Mouse AR, EPR, and EGF were assessed similarly, using commercially available sandwich ELISA assays or DuoSets (R&D Systems, Inc.).

2.3.6 Cell Death Detection ELISA

For measurement of apoptotic cell death in vitro, a quantitative cell death detection ELISA (Roche Applied Science, Penzberg, Germany) was performed. This assay used mouse monoclonal antibodies directed against DNA and histones to allow determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates. After incubation of cells with antibody drug for 24 hours, 100 μl of conditioned media was removed for growth factor ELISA analysis. The microtitre plate was then centrifuged at 200g for 10 minutes and the supernatant removed. The cell pellet was resuspended in 200 μl lysis buffer
and incubated for 30 minutes at room temperature. 20 μl of supernatant was transferred into the streptavidin coated assay plate for immediate analysis. 80 μl of immunoreagent was added to each well, the plate covered, and incubated with gentle shaking for 2 hours at room temperature. The solution was removed by suction and the plate washed 3 times with buffer. 100 μl of ABTS solution was added and the plate incubated with gentle shaking until colour development was sufficient for photometric analysis (approximately 10-20 minutes). Absorbance was measured at 405nm with the microplate reader.

2.3.7 Cell Proliferation Studies

A total of 3 x 10³ DU145, A431, and GEO cells were plated in 96-well plates with 200 μl media and grown to 75% confluence, then incubated with cetuximab in quadruplicate over a dose range of 0.01 – 100 μg/ml for 40-90 hours and viable cell count was then assessed using the MTS method. MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenol)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, is a tetrazolium compound that is bioreduced by cells into a formazan product that is soluble in culture medium \(^{175}\). A 1:20 mixture of phenazine methosulfate (PMS) PMS:MTS solution was prepared immediately prior to addition to culture plates. 20 μl of the combined solution was added to each well and plates were incubated at 37 °C in a humidified 5% CO₂ incubator for 1-4 hours. Absorbance was recorded at 490nm using the microplate reader Benchmark Plus (Bio-Rad Laboratories, Canada).
For the $^3$H-thymidine assay, A431 cells were prepared and treated as outlined above, then 2μCi of $^3$H-thymidine was added to each well. Cells were incubated for an additional 4 hours at 37 °C, then plates were freeze-thawed to lyse cells. Lysates were collected onto Unifilter-96 filter plates (Perkin Elmer, Waltham, MA) using a Filtermate filtration harvester (Perkin Elmer). Microscint-20 scintillation fluid (Perkin Elmer) was added to each well (25 μl), and a TopCount-NXT microplate liquid scintillation counter (Perkin Elmer) was used to evaluate cellular incorporation of $^3$H-thymidine.

Immediately prior to the MTS or $^3$H-thymidine assay 100 μl of medium was removed from each of the 4 wells and pooled for TGFα analysis by commercially available ELISA as outlined above.

2.3.8 RNA Extraction and Northern Blots

Cells were trypsinized, washed with PBS and centrifuged; pellets were resuspended in Trizol (Life Technologies Inc., Gaithersburg, MD). Trizol extraction was carried out according to manufacturer's instruction. RNA was DNase treated and quantified by U.V. absorbance. Human TGFα and TACE/ADAM17 cDNA fragments were generated by RTPCR, confirmed by sequencing, and used as a probe. Northern blotting was carried out with Dr. Giulio Francia as previously described 176. Twenty μg of total RNA were size fractionated on a 1% agarose-formaldehyde gel and transferred onto Hybond N filters (Amersham). Probes were $^{32}$P-dCTP labelled using an oligolabelling kit (Pharmacia Biotech) and hybridized to filters overnight at 42°C. Filters were
washed twice in 1 X SSC/0.1% sodium dodecyl sulphate (SDS) at room temperature for 15 minutes followed by 0.25 X SSC/0.1% SDS at room temperature for 15 to 30 minutes at 60°C and autoradiographed for 1 to 7 days. Glyceraldehyde-3-phosphate dehydrogenase was used as a loading control.

2.3.9 In Vivo Experiments

Female 8-10 week old Balb/C mice (Jackson laboratories, CA) were treated with 3 doses of ME1 or saline every 3 days by the intraperitoneal route. Plasma samples were obtained 24 hours after the third dose in Microtainer® plasma separating tubes (Becton Dickinson, Franklin Lakes, NJ), centrifuged at 7500g for 15 minutes at 4°C and frozen at -80°C until analysis for mouse-specific EGFR ligands as outlined above. Similar experiments were performed with erlotinib (100mg/kg), vandetanib (50mg/kg), or vehicle control given daily by oral gavage for 7 days, with plasma samples obtained 24 hours after the final treatment. For HT29 experiments 2 x 10^6 cells were injected into the peritoneum of 6-8 week old female athymic (nu/nu) mice (Harlan laboratories) and allowed to grow to a large tumour burden with ascites evident. Mice were then injected IP with a single dose of cetuximab, erlotinib, or saline. Animals were sacrificed 24 hours after injection and ascites samples were collected in Microtainer® plasma separating tubes and processed as for plasma samples. Growth delay experiments utilized HT29 cells that had been transfected with human chorionic gonadotropin β-subunit (β-hCG), as previously described to allow for non-invasive tracking of tumour burden in the urine of treated mice. For the growth
delay trial $2 \times 10^6$ HT29\textsuperscript{β-hCG} cells were injected into the peritoneum and cetuximab treatments were given at the doses indicated twice weekly beginning 6 days after tumour injection. Urine was collected from mice weekly using metabolic cages, pooled together within each treatment group ($n=5$) and frozen at -80 oC until analysis.

All experimental animal protocols in this thesis were approved by the animal care and use committee of the Sunnybrook Health Sciences Centre in accordance with the policies established in the Guide to the Care and Use of Experimental Animals prepared by the Canadian Council on Animal Care.

2.3.10 Mouse Angiogenesis Antibody Array

The following cytokines were included on the Transignal Mouse Angiogenesis Array (Panomics, Redwood City, CA): VEGF, TGF\textsubscript{α}, TGF\textsubscript{β}, EGF, FGF\textsubscript{α}, FGF\textsubscript{β}, G-CSF, Interleukins 1a, 1b, 4, 6, 12, leptin, TNF\textsubscript{α}, IFN\textsubscript{γ}, IFN-inducible protein 10, tissue inhibitor of metalloproteinase 1 and 2. All procedures followed the manufacturer's protocol: A 1:10 dilution of mouse plasma was incubated on a membrane containing immobilized antibodies allowing for the capture of the aforementioned proteins. After a wash step, the membrane was incubated with biotin-conjugated antibodies to detect captured proteins and, after incubation with streptavidin, electrochemical luminescence (ECL) was added for detection on scientific imaging film (Kodak, Blue XB-1, Rochester, NY). Results were reported as a comparison to positive (set to 100) and negative controls (set to 0) obtained via densitometry.
2.3.11 Densitometric Analysis

For analysis of the protein array membrane, exposures were imaged using a Molecular Imager GS-800 Calibrated Densitometer (BioRad, Hercules, CA) and quantified using Quantity One 1-D Analysis software (BioRad). Each dot representing an individual protein was standardized to relative background and compared to negative controls of that particular blot. Aggregated values for the treated groups were compared to aggregate values for the control groups. To ensure that quantification was performed in a linear range, three immunoblot exposures of varying intensity were analysed with only non-saturated blots subjected to quantification.

2.3.12 β-hCG and Urinary Creatinine Measurements

β-hCG was measured using a commercially available ELISA (Omega Diagnostics Ltd., Alva, Scotland, UK). After reagents were brought to room temperature, 50 ml of samples and standards were dispensed into wells of a microtitre plate, followed by 100 ml of buffer, then mixed for 30 seconds. The plate was incubated for 30 minutes in a wet box with some moistened paper towel. Microtitre wells were rinsed 5 times with distilled water and 150 μl enzyme conjugate reagent was added, followed by another, 30 minute incubation. 100 μl of substrate solution was added and the plate was incubated in the dark at room temperature for 20 minutes. The reaction was stopped with 100 μl stop solution and optical density determined at 450nm with the microplate reader within 10
minutes. β-hCG measurements were standardized for urine creatinine measured by colorimetric assay (Metra™, Quidel Corp, San Diego, CA). Urine samples, standards, and controls were diluted 1:40 with deionised water. 50 μl was added to the well of a microtitre plate and 150 μl of working colour solution (0.14% picric acid in sodium borate+SDS and 1N NaOH) was added. The mixture was incubated for 30 minutes at room temperature and optical density was read at 490nm using the microplate reader within 10 minutes of completion of the incubation step. Measurements were in agreement with an independent creatinine assay (Quantichrom™, Bioassay Systems, Hayward, CA). This model for tracking tumour burden with urinary β-hCG has been previously validated in our laboratory 177.

2.3.13 Patient Samples

Following institutional REB approval and informed consent, heparinised plasma samples were obtained from patients enrolled in a single agent cetuximab clinical trial for metastatic, EGFR-negative colorectal cancer. Cetuximab was administered at the standard dose regimen of 400mg/m² week 1, followed by weekly doses of 250mg/m². Blood samples were obtained 2 hours prior to, then 2 and 24 hours following the first, second and fourth treatment. Samples were centrifuged at 1000g for 15 minutes at 4°C, within 30 minutes of collection, and aliquots stored at -80°C until analysis.

Serum samples that were a part of a protocol from an ongoing phase I clinical trial of nimotuzumab were obtained from 3 patients. Samples were
obtained prior to and one day after each drug dose, which was administered on a weekly schedule. Samples were prepared and stored as described above, prior to analysis.

2.3.14 Statistical Analysis

Results of *in vitro* assays are reported as the mean ± SD, and *in vivo* assays and patient samples as mean ± SEM. Statistical significance was assessed by 1 way ANOVA, followed by Newman-Keuls or Dunnett's test, except in Figure 21, which was assessed by repeated measures ANOVA followed by Newman-Keuls test using GraphPad Prism© software package v.4.0 (GraphPad Software Inc., San Diego, CA). The level of significance was set at $P<0.05$. In figures: ***<0.001, 0.001<**<0.01, 0.01<*<0.05

2.4 Results

2.4.1 Dose-response effects are rapidly observed with anti-EGFR antibody but not RTKi treatment *in vitro*

To assess changes in ligand levels after drug treatment, human cancer cell lines that express EGFR were chosen from a wide variety of tumour types. We started by testing the PC3 cell line used previously for assessment of VEGF elevations following VEGFR-2 targeting with ramucirumab and CT322. After incubation with cetuximab, an increase in the TGF$\alpha$ ligand was observed, however the levels in conditioned media were very low (Figure 8). We therefore tested another prostate carcinoma cell line, DU145, as well as a mammary
carcinoma cell line MDA-MB-231 and found higher basal levels of TGF\(\alpha\), as well as higher increases with cetuximab treatment (Figure 8). Also similar to results with PC3 and VEGFR-2 targeted drugs, when proliferation and cell death were assessed in the DU145 cell line after incubation with a high dose of cetuximab, no significant alterations were observed (Figure 8), suggesting that drug treatment was not overtly harmful to these cells in vitro.

For further investigation, incubation with increasing doses of cetuximab for 24 hours revealed a dose-response effect on the level of TGF\(\alpha\) in conditioned media in many cell lines, which reached a plateau after a certain drug concentration had been reached (Figure 9). In addition, assessment of viable cell count using trypan blue stain did not reveal an appreciable difference with increasing drug dose, nor change the shape of the dose-response curve when ligand concentration was controlled for total viable cell count, as demonstrated in studies with the pancreatic carcinoma cell line BxPC3 (Figure 9). The cell lines reported collectively in figures 8 and 9 include PC3 prostate, DU145 prostate, MDA MB231 breast, BxPC3 pancreatic, HT29 colorectal, GEO colorectal, A431 epidermoid, and SW839 renal cell carcinoma, however TGF\(\alpha\) elevations were also observed in U87 glioma, and A549 lung carcinoma cell lines when these were tested. The level of TGF\(\alpha\) attained and the concentration of cetuximab at ligand plateau varied between cell lines. TGF\(\alpha\) was not the only ligand to demonstrate an increase with cetuximab, as a dose response was also noted for AR with the A431 cell line (Figure 9), however testing of AR in other cell lines, as well as other ligands, such as EGF and HB-EGF, revealed either non-
Figure 8 Treatment of PC3, DU145, and MDA-MB-231 with cetuximab in vitro. TGFα elevations were observed in the conditioned media after 24 hours of cetuximab incubation in each cell line (above). There was negligible impact of cetuximab on DU145 cell proliferation by MTS or cell death by ELISA (bottom).
Figure 9  Dose response for EGFR+ cell lines incubated with cetuximab for 24 hours. TGFα in conditioned media. Cell lines are listed with surface EGFR content mean fluorescence intensity by flow cytometry. BxPC3 reported with and without cell count. SW839 compared to IgG control. A431 also reported AR elevation.
detectable quantities by ELISA or levels that were conversely very high, and not
detectably altered by the addition of cetuximab.

To test how quickly the TGFα ligand elevation occurs, time-course studies
were performed using DU145 and HT29. The level of TGFα differed between
cetuximab-treated and untreated cultures as early as 15 minutes after drug
addition (the earliest time point studied), and accumulation of TGFα in
conditioned media became much more pronounced after 2 hours (Figure 10).
Similar experiments were performed with the RTKIs erlotinib, gefitinib, and
vandetanib. Upon incubation with RTKI at concentrations up to 100 μmol/l for 24
hours, no elevations in TGFα were observed compared to untreated cell cultures.

To test the potential effect of ligand affinity on TGFα elevation in vitro, 3
cell lines were tested with nimotuzumab, an EGFR neutralizing antibody with a
reported affinity that is 10 times lower than cetuximab. Results demonstrate the
requirement for much higher concentrations of nimotuzumab to increase TGFα in
these models, particularly for the high EGFR-expressing A431 cell line (Figure
11) where significant elevations in TGFα did not occur at the drug concentrations
tested.

2.4.2 Increased TGFα is not the result of transcriptional up-regulation and
is impacted by ligand cleavage

To investigate whether the TGFα increase could be the result of a
feedback response to EGFR signalling blockade resulting in increased TGFα
production, Northern blots were performed on lysates from the HT29, A431, and
Figure 10 Time course for TGFα ligand elevations in vitro. DU145 (top) and HT29 (bottom) were incubated with cetuximab at doses indicated. Close up view of early time points illustrated to the right.
Figure 11  Comparison of cetuximab and nimotuzumab for TGFα elevations in vitro. HT29, MDA-MB-231, and A431 cell lines were incubated with antibody drugs for 24 hours. TGFα was assayed in conditioned media by ELISA.
GEO cell lines incubated with cetuximab for 24 hours. No differences in TGFα mRNA after treatment were apparent, indicating that the increase in TGFα likely does not occur as a compensatory response to treatment at the transcriptional level (Figure 12A) during this time period. However, ongoing TGFα production is required to appreciate the maximum ligand effect during EGFR blockade. Experiments utilizing cycloheximide in HT29 demonstrated abrogation of the cetuximab-induced TGFα elevation in conditioned media (Figure 12B). The cycloheximide experiments were also performed in A431 cells, with similar results. Next, to examine the effects of cetuximab treatment on cell-associated TGFα protein, ELISA of cell lysates was performed. TGFα levels were decreased in lysates following treatment (Figure 12C), which prompted examination of the possibility that up-regulation of pro-TGFα cleavage was occurring. TGFα, like many receptor tyrosine kinase growth factors, exists as a pro-ligand attached to the plasma membrane that is cleaved by proteases for release of its active fragment \(^{178}\). The enzyme implicated in TGFα cleavage is TACE/ADAM-17 \(^{179}\). Inhibition of TACE activity using the MMP inhibitor TAPI-1 revealed near complete, but not total abrogation of the TGFα increase induced by cetuximab (Figure 12D) at 6 hours, demonstrating the important contribution of TACE to TGFα elevation during cetuximab treatment. The suppression was still evident at 54 hours, with levels approximately 90% lower than cetuximab treated cells with no TACE inhibition. Additionally, to rule out a compensatory increase in TACE production at the RNA level, Northern blots were performed
Figure 12  TGFα increases are not the result of transcriptional upregulation and are dependent on ligand cleavage by TACE. (A) Large increases in TGFα in conditioned media are observed after incubation with cetuximab for 24 hours in the HT29, A431, and GEO cell lines (top panel) but there is no detectable difference in TGFα mRNA in the cell lysates as assessed by Northern blot (bottom panel). Lanes of Northern blot correspond to the bars of graph directly above. (B) Cycloheximide treatment (100 μg/ml) suppresses the cetuximab-induced increase in TGFα to basal level in HT29 cells after 6 hours. (C) Cetuximab treatment leads to decreased TGFα protein in cell lysates as assessed by ELISA and expressed at pg/g total protein. Bars in bottom panel correspond to those in graph directly above. (D) TAPI-1 treatment (100 μg/ml) suppresses the cetuximab-induced increase in TGFα to near basal level in HT29 cells after 6 and 54 hours. Cetuximab dose = 100 μg/ml in all experiments. Symbols and bars, mean ± SD
and did not reveal a difference in TACE mRNA levels with and without exposure to cetuximab (Figure 12A).

Lysates from the cell lines analysed in figure 12 were also tested for AR, revealing much larger levels compared to TGFα. Interestingly, these cell-associated levels did also appear to drop upon treatment with cetuximab in the GEO and A431 cell lines (Figure 13). The A431 line, which contains a very high level of EGFR, was the one cell line where a dose-dependent increase in AR was demonstrated (Figure 9).

2.4.3 TGFα plateau predicts the optimal cetuximab concentration to inhibit tumour proliferation/viability in vitro

To assess whether the increase in ligands such as TGFα may have potential as a pharmacodynamic marker of optimal EGFR blockade, A431 and GEO cells, previously demonstrated to be sensitive to EGFR inhibition in vitro\textsuperscript{180,181}, were incubated with cetuximab in a dose-response study for 90 hours. When an aliquot of conditioned media was removed for TGFα analysis, followed by MTS assay, the maximum decrease in viable cell number corresponded to a steep increase in TGFα in conditioned media (Figure 14). Thymidine proliferation assays performed with A431 showed a similar result (Figure 14). Erlotinib caused a large reduction in viable cell number with this assay (approximately 50% at 40 hours) without a corresponding change in ligand levels in conditioned media compared to vehicle control.
Figure 13 Amphiregulin levels in cell lysates after cetuximab treatment. Decreases in AR protein in lysates as assessed by ELISA and expressed as pg/g total protein were observed after incubation of cells with cetuximab for 24 hours.
Figure 14  TGFα elevations predict antitumor efficacy in vitro. Maximum suppression of viable cell number in A431 and GEO cell lines is associated with a sharp increase in TGFα. Cells were plated in 96-well plates, incubated with cetuximab for 90 hours and assayed for viable cell number using the MTS method or tritiated thymidine. Media was pooled from 4 wells for TGFα analysis by ELISA immediately prior to beginning the MTS assay. Experiments conducted twice, in quadruplicate. Symbols and bars, mean ± SD.
2.4.4 TGFα ligand elevation shows a dose-response effect and parallels tumour suppression in a xenograft model

Given that the target of cetuximab is human EGFR, assessment of ligand changes *in vivo* necessitated the implantation of a human tumour xenograft into immunodeficient mice. In an attempt to obtain sufficient growth factor levels for assay by ELISA, a number of human tumour cell lines were studied. The approach was similar to that taken with the prostate carcinoma cell line PC3 and VEGFR-2 targeted drugs, i.e. to examine tumours implanted into the peritoneal cavity for development of ascites, then to assess growth factor levels circulating in plasma and the ascites fluid. While PC3 tumours did not produce detectable EGFR ligands in ascites fluid, there were detectable quantities of TGFα in DU145 prostate carcinoma and Caki-1 renal cell carcinoma xenografts (Figure 15). Although quantities detected in DU145 were low, time course studies demonstrated a rapid elevation, similar to what was seen in the PC3 system with VEGF, and no elevations were detected with either cell line upon use of a non specific IgG antibody (Figure 15).

To investigate the potential predictive ability of TGFα elevations, a monitoring system to track tumour burden within the peritoneal cavity during cetuximab treatment was required. HT29 colorectal cancer cells were utilized that had been transfected with human chorionic gonadotropin β−subunit (β-hCG). This secreted artificial tumour marker was previously developed and validated by Shih *et al* 182, and utilized successfully in the HT29 intraperitoneal ascites tumour model in our laboratory 177. Plasma levels of TGFα were not detectable using
Figure 15  TGFα in ascites fluid from IP DU145 and Caki-1 xenografts treated with cetuximab. Time course study performed in DU145 cells at high intraperitoneal tumour burden after a single 1000 μg/mouse IP dose of cetuximab or IgG. Three mice/group. Symbols, mean +/- SEM. Caki-1 confirmatory experiment performed by scientists at Imclone systems, Inc., in which ascites fluid samples were obtained 24 hours after a single dose of saline, IgG, or cetuximab.
this model; however ascites fluid samples provided sufficient growth factor for detection. This tumour demonstrated a TGF\(\alpha\) dose-dependent increase and plateau at the \(\geq 500 \mu g\) cetuximab dose 24 hours after a single treatment (Figure 16). This effect was not observed after treatment with the RTKI dual EGFR and VEGFR inhibitor vandetanib (dose of 50mg/kg IP). In addition, when controlled for tumour burden/cell count using the \(\beta\)-hcg as a surrogate marker, measured in both plasma, and ascites fluid, the increases in TGF\(\alpha\) remained at the \(\geq 500 \mu g\) dose level (Figure 16). Confirmatory experiments incorporated the lower affinity antibody nimotuzumab and the EGFR RTKI erlotinib (dose of 50mg/kg IP). Interestingly, but perhaps not surprisingly, no increase in TGF\(\alpha\) was observed with erlotinib, and a small but insignificant elevation (\(p=0.11\) by unpaired student’s t test) was detected with a high dose of nimotuzumab (Figure 17), reflecting what had been observed with in vitro models.

A growth delay trial was performed in this model with cetuximab dosing twice weekly IP beginning 6 days after tumour cell inoculation. Tumour burden assessment via urinary \(\beta\)-hCG measurements was obtained weekly. \(\beta\)-hCG began to increase between weeks 4 and 5, and by week 6 the 500\(\mu\)g and 1000\(\mu\)g cetuximab dose groups showed the least tumour burden by urinary \(\beta\)–hCG measurement (Figure 17). Optimal efficacy had been predicted for these dose groups by TGF\(\alpha\) elevation in prior studies (Figures 16 and 17). This xenograft model also results in primary tumour growth in the subcutaneous tissues at the site of IP injection (so-called transperitoneal growth), and the size of these nodules has been demonstrated previously to correspond to overall
Figure 16  TGFα dose response study in ascites of HT29.hcg IP xenografts. Cetuximab administered IP at doses indicated. Vandetanib given as a single dose of 50mg/kg PO. Ascites sample taken 24 hours after treatment and assayed for TGFα by ELISA. TGFα levels also controlled for tumour burden using β-hcg expression in plasma and ascites fluid, with similar result. 4 mice/group. Bars, mean + SEM. 1-way ANOVA with Dunnett’s test.
Figure 17 Increased TGFα in ascites fluid correlates with antitumor efficacy of cetuximab in a preclinical xenograft model. (A) HT29 cells transfected with β–hCG show a TGFα dose-response relationship 24 hours after a single dose of cetuximab. Minimal elevation was seen following nimotuzumab or erlotinib treatment (50mg/kg). 1 way ANOVA with Dunnett's test for multiple comparisons. (B) β–hCG in urine of mice bearing HT29β-hCG intraperitoneal xenografts and treated with cetuximab twice weekly at the doses indicated (µg/mouse), beginning 6 days after tumor implantation. (C) Urine was pooled from mice in each group for β-hCG analysis and normalized for creatinine; tumour burden was also assessed by measurement of growth at injection site. 4 mice/group. Symbols and bars, mean +/- SEM.
tumour burden\textsuperscript{177,183}. Indeed, when plotted over time, the 500 and 1000 \(\mu\)g dose levels had the smallest burden when measured using this method; however the separation between groups was less striking than the use of the urinary \(\beta\)hcg marker (Figure 17).

### 2.4.5 Measurement of other ligands in the HT29.hcg IP xenograft model

Consistent with results from in vitro experiments that showed high levels of the low affinity ligand AR in many cell lines, including HT29, the ascites fluid from the HT29.hcg IP model contained high levels of AR, which became even more elevated upon treatment with either cetuximab or nimotuzumab (Figure 18). Despite these high levels, this human AR was not detectable in mouse plasma by ELISA. Interestingly, while levels were much lower than both AR and TGF\(\alpha\), EGF was detectable and elevations at high cetuximab doses were observed in ascites fluid (Figure 18). When the in vivo and in vitro data was taken together, ligand results with TGF\(\alpha\) appeared most promising as a potential biomarker for patients treated with cetuximab, however alterations in other ligands, such as AR and EGF were observed in certain tumour cell lines.

### 2.4.6 Plasma ligand elevations occur following anti-EGFR antibody but not RTKi treatment of non tumour-bearing mice

Previous experiments utilized human tumour xenografts to investigate the changes in ligand in response to treatment with drugs targeting human EGFR. Data with anti-mouse VEGFR-2 antibodies and RTKIs had shown that the
Figure 18 AR and EGF levels in ascites after treatment with cetuximab or nimotuzumab. HT29-hcg tumour cells were implanted into the peritoneal cavity of nude mice and cells grew to a high tumour burden with ascites evident. A single dose of drug was administered IP at doses indicated, and ascites was sampled after 24 hours. Growth factors were measured by ELISA. 4 mice/group. Bars, mean ± SEM. 1-way ANOVA with Dunnett’s test for multiple comparisons.
biomarker was associated with a host response, as VEGF elevations could be produced in normal mice \(^{44,45}\). To assess the potential contribution of host EGFR-positive tissue blockade, an anti-mouse EGFR neutralizing antibody was required. Very fortunately, such an antibody, called ME1 became available in sufficient quantity to treat non tumour-bearing mice. Normal BalbC mice were treated with 3 IP injections of ME1 over 1 week, followed by plasma sampling 24 hours after the 3\(^{rd}\) dose. Plasma samples taken from these mice revealed increases in circulating AR and EPR, but not EGF (Figure 19). When mice were treated with high doses of erlotinib by daily gavage for 1 week there were no significant increases in any of these ligands (Figure 19, similar results to erlotinib were obtained for the RTKI vandetanib). For confirmation of the effect of species specificity, cetuximab (which only binds human EGFR) was included as a negative control, and did not produce an increase in any of the ligands tested (Figure 19). Following demonstration of ligand increases in the plasma of non tumour-bearing BalbC mice treated with ME1, a dose response evaluation was performed. Both plasma AR and EPR showed dose-dependent increases, with AR reaching higher levels in the circulation, while EPR showed a more significant increase over baseline at each dose level studied, and EGF showed no increase (Figure 19).

As there was no commercially available ELISA, nor recombinant protein or antibody components to construct one, assessment of mouse TGF\(\alpha\) was carried out using a commercially available mouse antibody array, that analysed a number of proteins important to angiogenesis, including VEGF and EGF.
Figure 19 Anti-mouse EGFR antibody ME1 but not the RTKI erlotinib causes an increase in plasma AR and EPR in BalbC mice. Mice were treated with daily oral gavage of erlotinib (100mg/kg) or vehicle for 7 days, or intraperitoneal injection on days 1,4,7 of cetuximab (1mg), ME1 (1mg) or saline (5 mice/group). Plasma samples were obtained 24 hours after the final treatment and assayed for AR, EPR, and EGF by ELISA (top). ME-1 induced ligand increases observed for AR and EPR in normal mice are dose-dependent (bottom). Bars, mean + SEM. 1-way ANOVA with Dunnett’s test, except t-test for erlotinib vs. vehicle.
Results from this array demonstrated a 3.2 fold increase in TGFα in ME1 antibody-treated vs. control (Figure 20). Although less quantitative, this assay confirmed the elevated TGFα results that were demonstrated using in vitro xenograft models and incorporated a different methodology. The array also confirmed the low levels of EGF in mouse plasma both before and after cetuximab treatment, as they were undetectable in this assay. Interestingly, changes in other cytokines assessed in this array included increases in acidic FGF, leptin, and TNFα, as well as a decrease in VEGF; the VEGF result being consistent with previous findings associated with anti-EGFR antibody therapy.

2.4.7 Plasma TGFα elevations are observed in patients following cetuximab treatment

For assessment of circulating ligand changes in a clinical setting, plasma samples were obtained from 6 patients undergoing single agent cetuximab treatment for EGFR-negative metastatic colorectal cancer. These patients received cetuximab at the standard dose regimen of 400mg/m² IV on week 1, followed by weekly doses of 250mg/m² IV until disease progression or unacceptable toxicity. Levels of TGFα were below the limit of detection in 3 of 6 patients prior to the first dose, however TGFα levels increased significantly by the second week of therapy in all 6 patients, and remained significantly elevated at week 4 (Figure 21). The only ligand to show a consistent increase in all 6 patients was TGFα; as plasma AR and EGF, while detectable in all 6 patients, did
Figure 20  Angiogenesis array demonstrating elevated TGF\(\alpha\) in plasma of mice treated with ME1. Results with other cytokines in the array included apparent elevations in FGF\(\alpha\), leptin, TNF\(\alpha\), and decreased VEGF. EGF was not detected by this assay (pooled plasma from \(n=5\) mice per group). EGFR antibody dose and schedule 1 mg day 1,4,7 IP. Bars, mean + SD from 2 readings.
not reveal a significant increase, although certain individuals did show elevations with cetuximab treatment (Figure 21). Finally, EPR, HB-EGF and betacellulin (BTC) levels were detectable in only 3 out of 6 patients, but increased upon cetuximab treatment in some cases. Notably, circulating VEGF, which is not a direct ligand for EGFR, but is positively impacted by EGFR signalling, appeared to mildly decrease in patients treated with cetuximab (Figure 21), which is consistent with prior studies [145,184-186]. Finally, a subset of patients had plasma samples obtained 2 and 24 hours after the first treatment to determine how quickly plasma ligand levels begin to change. Results with TGFα showed that circulating levels of this ligand begin to elevate within hours of treatment (Figure 21).

2.4.8 Pilot study of 3 patients treated with nimotuzumab

To investigate whether TGFα, AR, and/or EGF would be consistently elevated upon treatment with the lower affinity antibody nimotuzumab, left-over serum that had been collected from 3 patients involved in a phase I trial was analyzed. Samples were obtained just prior to treatment, and 24 hours later on weeks 1 and 2 of a weekly treatment regimen. Each of the 3 patients had been treated with a different nimotuzumab dose: 50, 100, or 200 mg. Plasma samples were not obtained in this study. Results for TGFα were below the limit of detection in all 3 patients at all 4 time points. However, AR levels were detectable and appeared to increase within 24 hours of each treatment, and decrease slightly between doses (Figure 22). Although detectable, and in some
Figure 21  Cetuximab treatment causes increased plasma TGFα in EGFR-negative colorectal cancer patients. Plasma samples obtained from patients prior to the 1\textsuperscript{st}, 2\textsuperscript{nd}, and 4\textsuperscript{th} cycle of treatment and assayed for TGFα, AR, EGF, and VEGF by ELISA. Samples also obtained after 2 hours in 5 patients and 24 hours in 2 patients. Symbols, mean +/- SEM. Repeated measures ANOVA with Newman-Keuls post test for multiple comparisons.
Figure 22  Serum AR and EGF in 3 patients treated with nimotuzumab. Weekly IV treatments were given at the doses indicated. Samples were obtained prior to and 24 hours after the 1st two cycles of treatment.
cases in very large quantities, serum EGF did not show a consistent pattern of change (Figure 22), although it must be emphasized that this was a very small patient population. Nonetheless, given the data obtained with both EGFR antibodies, it would seem unlikely that the consistent TGFα elevations observed with cetuximab treatment would be expected with nimotuzumab. Conversely, and consistent with other preclinical data, the lower affinity ligand AR could potentially be displaced/elevated by either antibody drug, although this result was not observed in the 6 cetuximab treated patients.

2.5 Summary

Using a number of EGFR-positive tumour cell lines in vitro we observed an increase in TGFα in a dose-dependent manner following treatment with cetuximab, but not with any of the RTKIs. Neither transcriptional up-regulation of TGFα mRNA, nor that of the metalloproteinase TACE, which is responsible for pro-TGFα cleavage at the plasma membrane, was associated with the increases observed. The ligand plateau did, however, correlate with the optimal concentration of cetuximab required to inhibit A431 and GEO viable cell proliferation in vitro. In addition, TGFα elevations in the ascites fluid from intraperitoneal HT29 xenografts correlated with optimal tumour growth delay. When administered to normal mice, an anti-mouse EGFR neutralizing monoclonal antibody produced increases in plasma TGFα, AR, and EPR, reflecting the contribution of host tissues to this effect in vivo. Again, these increases were not observed with the use of an EGFR-specific RTKI. Finally, in
a clinical pilot study, significant increases in plasma TGF\(\alpha\) during cetuximab treatment were demonstrated in 6 EGFR-negative colorectal cancer patients, and therefore could represent a useful aid to optimal dose determination for this agent in clinical trials, if these data were confirmed with higher patient numbers. With nimotuzumab, the potential for TGF\(\alpha\) to serve as this kind of a pharmacodynamic biomarker would appear less likely, given the preclinical data, and possibly the lack of elevation detected in 3 treated patients, although again, this lack of effect must also be confirmed with larger patient numbers before conclusions can be made.

2.6 Discussion

2.6.1 Use of ligand(s) as a clinical biomarker

The study of potential biomarkers for application to EGFR targeted drugs has focused mainly on predicting which patients will respond favourably to treatment, and the use of sequential biopsies with assessment of signalling proteins histologically has shown some promise in this regard\(^{180;187}\), as have k-ras mutations\(^{174;188}\). Additionally, EGFR ligands have been studied in tumour tissue as prognostic markers for treatment response and/or survival in both experimental models and clinical trials. Interestingly, high levels of tumour AR and EPR gene expression from both primary tumour and metastases were associated with a positive response to cetuximab in recent colorectal cancer trials\(^{174;189}\). While it is currently unknown whether or not the serial assessment of ligand changes would be useful for individual patient prognosis, this test may
have utility as a pharmacodynamic marker indicative of adequate drug exposure. The use of such a biomarker, as investigated in this thesis, has a unique purpose, namely optimizing conditions of target blockade which in turn is hoped to optimize the potential for response, realizing that there will likely be patients with tumours that show inherent drug resistance despite optimal drug application. Establishment of a standard dose regimen based on pharmacokinetics is an important component of clinical drug application. What monitoring of ligand elevations could provide, if successful, in addition to confirmatory evidence for the chosen regimen, would be a real-time marker of receptor occupancy by antibody in each individual patient. This could allow for alterations from the standard dose, similar to what is done with traditional chemotherapeutic regimens, using toxicities such as myelosuppression. A patient that does not exhibit increased ligand upon beginning treatment may benefit from dose escalation, for example. Attractive features of ligand monitoring in the circulation, when compared to other biomarkers, include the ease of application of ELISA technology and the non-invasive nature of repeated sample acquisition.

2.6.2 Tissue distribution of ligand changes

EGFR signalling is prevalent in many tissues, and the side effect profile of EGFR inhibitors reflects this fact. Increases in serum or plasma TGFα in patients may therefore reflect the degree of EGFR blockade throughout the entire patient, i.e. both normal and tumour tissue, which is why it is not surprising to observe this increase in non tumour-bearing mice, as well as
patients with EGFR-negative tumours, the difficulties inherent in proper tumour EGFR classification not withstanding. It follows from this rather holistic view that assessment of the other EGFR ligands in circulation may give a more complete picture of EGFR blockade. It is also possible that the specific ligand(s) of importance differ between different tumours as well as various normal tissues associated with EGFR-targeting, such as keratinocytes vs. intestinal epithelia for example. While cutaneous rash is a host tissue response that has demonstrated potential as a predictive factor for response to EGFR inhibitors as well as survival in some studies, assessing the effectiveness of EGFR blockade using the severity of cutaneous side effects reflects only one tissue type, whereas it is possible that circulating ligand changes may be more reflective of total body EGFR. It would be of interest to undertake parallel studies comparing extent of TGFα and other ligand increases with the severity of rash.

2.6.3 Differences between antibodies and RTKIs

In contrast to cetuximab, none of the three EGFR-targeted RTKI’s evaluated produced an elevation in EGFR ligands in conditioned media or plasma, however the treatment time was relatively short, and the possibility remains that changes develop with more prolonged therapy. Additionally, these RTKI’s have a much shorter half-life than antibodies, which would allow growth factor level increases in the circulation, if they occur, to return to baseline levels very rapidly as circulating drug levels fall. This has previously been demonstrated with VEGF after treatment with RTKI’s such as sunitinib. However, in the context of EGFR
targeting, results of a recent phase II colorectal cancer clinical trial with gefitinib did not reveal a reliable increase in serum TGFα during treatment \(^{200}\), which is consistent with our results. It would be incorrect to conclude that circulating TGFα never increases in patients treated with gefitinib, however, as this trial demonstrated an increase in 6/16 (38%) after 1 week in patients treated with a 500mg/day dose, and 5/12 (42%) in those treated with 250mg/day. There was no association between increase in TGFα and progression-free survival in that study. Changes in TGFα have also been documented during gefinitib treatment in other tumor types. A recent study of 250mg daily gefinitib treatment of head and neck squamous cell carcinoma demonstrated an increase in 15/37 patients, a decrease in 19 and no change in 3 patients after 8 weeks of therapy \(^{201}\). Interestingly, patients with a partial response or stable disease trended toward a greater increase in TGFα, although there was no association with progression-free survival or overall survival. Conversely, in non-small cell lung cancer patients, a recent trial that assessed serum TGFα levels before gefitinib treatment, showed shorter survival times in patients considered “TGFα-positive” \(^{202}\). These studies appear to reveal a tumor-dependent relationship of circulating TGFα and outcome; one that may contrast the host-tissue dependence observed in our experiments. Future trials with cetuximab and other EGFR monoclonal antibodies may shed further light on whether TGFα or other ligands will become increased in nearly all treated patients when given at an optimal dose, and/or also serve as an accurate predictor of response to treatment.
Furthermore, regarding cetuximab, a recent case report demonstrated increased TGFα in a patient with Menetrier’s disease\textsuperscript{203,204}.

Other ligands were not reported, however the authors concluded that the TGFα elevation, detected in both serum and gastric juice 24 hours after a single treatment, was evidence of effective receptor blockade. This conclusion is also consistent with the fact that neutralizing antibodies such as cetuximab have been utilized as tools to study autocrine EGFR signalling\textsuperscript{205}. Prior to the use of a neutralizing antibody, it was difficult to study growth factor levels in conditioned media, as TGFα for example, would be quickly internalized after binding EGFR\textsuperscript{178}. As receptor binding and internalization represents a major clearance mechanism for this growth factor\textsuperscript{138,206}, disruption of this clearance by cetuximab results in free TGFα accumulation, regardless of whether or not the growth factor source is autocrine in nature. Conversely, the small molecule ATP-mimetics, which bind inside the cell, continue to allow ligand to engage surface EGFR. Therefore, in addition to their vast differences in pharmacokinetics, the discrepancy between EGFR antibodies and RTKIs could also be related to these differing sites of drug binding. Perhaps the most gratifying clinical result reported with cetuximab, in addition to the case report of Menetrier’s disease, has been the recent documentation of increased plasma TGFα in 73% (29 of 40) rectal cancer patients that received capecitabine and cetuximab (400mg/m\textsuperscript{2} initial dose) 1 week prior to chemo-radiation treatment\textsuperscript{207}. Interestingly, plasma EGF levels were not significantly altered, and circulating EGFR was decreased, which again resembles previous findings with soluble VEGFR-2. Increased TGFα levels also
correlated with T downstaging in this study, which suggests that this biomarker may, in addition to playing a pharmacodynamic role, serve as an indicator of tumour response\textsuperscript{207}.

2.6.4 Mechanistic considerations

TGF\(\alpha\) was the only ligand in this study that became consistently elevated upon EGFR antibody treatment and would therefore appear to be the most promising for potential application as a biomarker, although the number of patients assessed in this pilot study was small. Potential reasons for the observed variability between ligands include differences in receptor affinity and/or intracellular trafficking\textsuperscript{139;140;208}. Further studies are needed to investigate the role such factors may have in treatment-induced ligand changes. TGF\(\alpha\) is also considered to be a dissociative ligand that is released from EGFR in the acidic environment of the endosome, unlike EGF that is targeted for receptor-bound degradation\textsuperscript{139;140;208}. This dissociative nature may allow for at least some recycling\textsuperscript{139}, which could further increase the levels of circulating TGF\(\alpha\) in an environment of EGFR blockade. This inherent difference in the way various EGFR ligands are processed upon internalization could determine whether certain EGFR ligands may prove to be useful as circulating biomarkers.

Other changes occurring with treatment could affect the magnitude of TGF\(\alpha\) elevation over time, including the down-regulation of surface EGFR levels that occurs following treatment with antibodies like cetuximab\textsuperscript{209}. This process takes hours to develop, but ultimately leads to a decrease in the total amount of
target available. Trafficking studies have recently been published with Mab225, the precursor antibody to cetuximab, demonstrating a 30-40% decrease in surface EGFR as well as a decrease in total EGFR levels in A549 and HeLa cells \(^{209}\). Conversely, it appears that small molecule inhibitors may not alter the total amount of EGFR during treatment \(^{209}\). In conclusion, the rapid and dose-dependent nature of these results, combined with the lack of compensatory up-regulated production or cleavage supports a “displacement” hypothesis that results in a transient suppression of TGF\(\alpha\) clearance. The resulting increase in circulation is perhaps exacerbated by other factors such as ongoing tissue TGF\(\alpha\) production, dissociative intracellular trafficking/recycling and treatment-related EGFR down-regulation.

The inability of TGF\(\alpha\) to be cleared after blockade of its normal route of internalization does not address the question of whether or not production or cleavage becomes up regulated as a result of EGFR blockade. Our Northern blot results would appear to refute the possibility of compensatory upregulation. A positive feedback autocrine loop has previously been described for the A431 cell line, among others \(^{174;210;211}\). Treatment with cetuximab resulted in a reduction of TGF\(\alpha\) mRNA at four hours \(^{210}\), so it is not surprising that there was no compensatory increase at the transcriptional level in our experiments. Ligand cleavage activity was down regulated upon disruption of EGFR signalling in that same study \(^{210}\). The very rapid onset of differences in TGF\(\alpha\) between treated and untreated cell cultures may also be significant mechanistically, as it decreases the likelihood that up-regulated growth factor production is responsible
for these results. However, the increase in circulating levels of TGFα is perhaps exacerbated by other factors such as ongoing constitutive tissue TGFα production and/or decreased cell surface EGFR expression that has been demonstrated with cetuximab treatment\(^{209}\).

Certain EGFR ligands may bind more than the receptor being targeted by monoclonal antibody; EPR, HB-EGF, and BTC can all bind both EGFR and HER4 for example\(^{178}\). The biological implications of increases in circulating levels of these ligands secondary to antibody treatment that targets only EGFR remain a priority for investigation. The increase in ligands considered “off target”, such as FGFα, Leptin, and TNFα is also of interest. A related finding, but with differing cytokines, was recently demonstrated with sunitinib\(^{45}\). The mechanisms underlying such effects are unknown. The clinical utility of elevated circulating TGFα, as well as other EGFR ligands, as potential pharmacodynamic biomarkers during EGFR-directed antibody treatment is suggested by our data, and serial ligand assessment should be included in the design of future clinical trials.
Chapter 3

Impact of EGFR Inhibitors on “Off Target” Ligands, CEPs, and the Interaction with Chemotherapy

3.1 Abstract

The EGFR signalling pathway has been implicated in angiogenesis in many ways, including through its influence on the potent endothelial growth and permeability factor VEGF. Inhibitors of VEGF receptors cause alterations in circulating growth factors considered off target, and some of these factors, along with VEGF, are potent inducers of CEPs. We performed experiments to examine whether EGFR targeted drugs could also impact off target growth factors and CEPs in circulation. Normal BalbC mice were treated with injections of saline or the anti-mouse EGFR monoclonal antibody ME1 every 3 days, or daily gavage of vehicle or the EGFR RTKIs erlotinib, vandetanib or lapatinib. Plasma levels of the off-target ligands G-CSF, SDF1α and SCF were evaluated, as these growth factors have showed modulation with the use of VEGF inhibitors in previous studies. Viable CEPs were enumerated using four-colour flow cytometry. The EGFR-targeted antibody ME1, as well as the EGFR RTKIs erlotinib, vandetanib and lapatinib, caused increased circulating G-CSF at higher doses, but no consistent alterations in the other growth factors were observed. Increased circulating VEGF was also observed with higher doses of ME1 and lapatinib. A dose-dependent decrease in CEP levels occurred with ME1 and lapatinib. However, CEPs were not decreased at higher drug doses associated with
increases in circulating VEGF and G-CSF. Finally, cyclophosphamide and irinotecan chemotherapy treatment was tested alone and in combination with ME1. No significant changes in the level of on target ligands amphiregulin or epiregulin were observed with chemotherapy treatment alone. Cyclophosphamide partially attenuated the elevation in circulating amphiregulin that is observed with ME1 when these drugs were used in combination. In conclusion, treatment of normal mice with EGFR inhibitors at high doses leads to increased circulating G-CSF. Viable CEPs may also be suppressed in circulation with EGFR inhibitors at certain doses. However, this suppressive effect was not observed at the higher doses that increased circulating G-CSF and VEGF.

3.2 Introduction

The link between EGFR signalling and angiogenesis, via VEGF signalling is well established. Indeed, our data from a pilot study in patients treated with cetuximab reported in the previous chapter illustrates the potential impact that EGFR inhibition may have on decreasing circulating VEGF levels, as has been documented by others. We have also analysed VEGF from HT29 cells treated with cetuximab in the monolayer culture model and found decreases in VEGF in conditioned media (Figure 23). VEGF is a potent inducer of CEP mobilization, and may have a profound effect modulating systemic vasculogenesis – the contribution to blood vessel growth made by circulating cells originating in the bone marrow. EGFR targeted drugs may influence CEPs, which may be another mechanism by which these agents contribute to inhibiting
Figure 23 Simultaneous assessment of TGFα and VEGF in HT29 cells incubated with cetuximab. Conditioned media was assayed for growth factors by ELISA after 24 hours of incubation with cetuximab. Symbols and bars, mean + SD.
angiogenesis. The acquisition of an anti-mouse EGFR neutralizing antibody (ME1) permitted the studies in this chapter to be conducted in non-tumour bearing mice. We chose BalbC mice for this work because this strain has relatively high basal levels of CEPs in circulation, which permits the assessment of CEP changes in either direction upon drug treatment.

VEGF is not the only growth factor known to affect CEP recruitment. Other factors, such as G-CSF and/or SDF1α have also been demonstrated to have a role in mobilizing these cells. Given that these growth factors have been shown to be increased following treatment with anti-angiogenic RTKIs, such as sunitinib, we chose to undertake assessment of these factors in response to EGFR blockade. If EGFR inhibitors affect systemic vasculogenesis via CEP modulation, the nature of the CEP changes could be influenced by any of these cytokines, or possibly as yet unrecognized growth factors. Finally, given that cetuximab, panitumumab, and other EGFR antibodies are frequently used in conjunction with systemic chemotherapy, we wished to assess whether the chemotherapy drugs modify growth factor ligand responses, and whether their presence should be taken into consideration when circulating growth factor levels are investigated.

3.3 Methods

3.3.1 Reagents and Drugs

The anti-murine EGFR neutralizing antibody ME1 was provided by Imclone Systems Inc. (New York, NY) and was diluted in 0.9% sterile normal
saline before intraperitoneal injection in vivo. The activity of the anti-murine EGFR neutralizing antibody has been described previously. Erlotinib (Tarceva®) was generously provided by Genentech (San Francisco, CA). Vandetanib (Zactima®) was generously provided by Alan Barge at AstraZeneca (Macclesfield, UK). Lapatinib was generously provided by Glaxo-SmithKline, (Philadelphia, PA). Erlotinib and vandetanib were given by p.o. gavage of homogeneous suspension in 1% Tween 80 in saline. Cyclophosphamide, Irinotecan (CPT11)

3.3.2 CEP analysis

Blood samples were obtained from the orbital sinus of 10 week old BalbC mice. CEP evaluation was performed with Dr. Ping Xu by enumeration using four-colour flow cytometry (BD FACSCaliber). Monoclonal antibodies reacting with CD45 were used to exclude hematopoietic cells; CEPs were depicted using the endothelial murine markers VEGF receptor 2 fetal liver kinase 1, CD13, and CD117 (Pharmingen BD, San Diego, CA). Nuclear staining (Procount; BD Biosciences, San Jose, CA) was used to exclude the possibility that platelets or cell debris hampered the accuracy of CEP enumeration. After RBC lysis, cell suspensions were evaluated using analysis gates designed to exclude dead cells, platelets, and debris. After acquisition of at least 100,000 cells/sample, analyses were considered as informative when adequate numbers of events (i.e. >50, typically 100-200) were collected in the CEP enumeration gates. Percentages of stained cells were determined and compared with negative
controls. Positive staining was defined as being greater than nonspecific background staining, and 7AAD was used to enumerate viable, apoptotic, and dead cells.

### 3.3.3 In Vivo Experiments

Female 8-10 week old Balb/C mice (Jackson laboratories, CA) were treated with 3 doses of ME1 (500 μg dose was used in all non-dose response experiments) or saline every 3 days by the IP route. Plasma samples were obtained 24 hours after the third dose in Microtainer® plasma separating tubes (Becton Dickinson, Franklin Lakes, NJ), centrifuged at 7500g for 15 minutes at 4°C and frozen at -80°C until analysis for mouse VEGF, PI GF-2, G-CSF, SDF1α, SCF, and AR by ELISA (R&D Systems, Inc). Similar experiments were performed with erlotinib (100mg/kg), vandetanib (50mg/kg), or vehicle control given SID by oral gavage for 7 days, with plasma samples obtained 24 hours after the final treatment. ELISA protocols were identical to those performed in the previous chapter, with the following modification from manufacturer’s instructions: For mouse VEGF, a 1:2 dilution was made instead of the recommended 1:5.

### 3.3.4 Statistical Analysis

Results of in vivo assays are reported as mean ± SEM. Statistical significance was assessed by 1 way ANOVA, followed by Newman-Keuls or Dunnett's test, using GraphPad Prism© software package v.4.0 (GraphPad...
Software Inc., San Diego, CA). The level of significance was set at $P<0.05$. In figures: ***$<0.001$, $0.001<**<0.01$, $0.01<*<0.05$

3.4 Results

3.4.1 Effect of EGFR drugs on “off target” ligands

We chose a select number of off-target cytokines to assess in response to EGFR targeted agents. In addition to VEGF, P/LGF is a growth factor that is involved in angiogenesis, binds to VEGF receptor-1, and has been shown to increase in response to VEGF receptor-targeted drugs. In addition, the non-VEGF receptor binding growth factors G-CSF, SDF1$\alpha$, and SCF were each evaluated. These growth factors were chosen because they can promote angiogenesis, and their levels are altered with the use of targeted drugs such as sunitinib $^{45}$. When normal BalbC mice were treated with 3 injections of saline or ME1 over 1 week, or daily gavage of vehicle or the EGFR RTKIs erlotinib or vandetanib, no clear pattern of changes emerged, with a few exceptions. First, erlotinib treatment at a dose typically used in preclinical studies resulted in elevated circulating G-CSF (Figure 24). Vandetanib also increased G-CSF, but not to a significant level in these experiments, although it has been reported to do so in another study $^{45}$. Vandetanib is a dual inhibitor of VEGFR and EGFR, and would appear to have served as a positive control for VEGF, P/LGF, and SDF1$\alpha$-related elevations (Figure 24), given that these changes have been described previously for this drug, and attributed to its VEGF-inhibiting effects $^{45}$. It is not known whether this spectrum of growth factor changes results from the drug’s
Figure 24  Plasma levels of “off target” growth factors after treatment of normal mice with EGFR inhibitors. 10 week BalbC mice were treated for 1 week with daily PO gavage of RTKI or 3 doses of antibody (Day 1,4,7) IP. Plasma samples were obtained 24 hours after treatment and mouse VEGF, PIGF2, G-CSF, SDF1α, and SCF were assayed by ELISA. Bars, mean + SEM.
VEGF or EGFR blockade, however similar changes with other VEGFR inhibitors, such as sunitinib, coupled with the lack of at least some of these changes in EGFR-restricted inhibitors, such as erlotinib, would suggest the elevations are related to VEGF pathway disruption. Cetuximab, which does not bind murine EGFR, was utilized in these experiments as a negative antibody control, and did not reveal any significant alterations from saline treatment in these mice.

Following the survey of antibody and RTKI drugs for changes in plasma levels of growth factors, a dose-response study was conducted with ME1. Results showed no change, or possibly a decrease in circulating VEGF with increasing ME1 dose, until the dose level of 1000 μg was reached, where VEGF levels did become elevated (p=0.03 by 1 way ANOVA) (Figure 25). This VEGF increase was not accompanied by a corresponding dose-dependent change in circulating PIGF levels. Similar to erlotinib, when an ME1 dose response was analysed for G-CSF there was a significant trend to elevation with dose, which became significant over saline control mice at the highest dose level (p=0.012 by 1 way ANOVA, post test for linear trend p=0.0009) (Figure 25). There were no significant increases in mouse SDF1α or SCF, apart from elevated SDF1α in the 500 μg dose group.

Finally, plasma from mice treated with the dual EGFR/HER2 RTKI lapatinib was tested for circulating AR, as well as the off-target growth factors VEGF and G-CSF. This drug was given to mice PO as a once daily (SID) or twice daily (BID) gavage for 4 weeks. Plasma samples were analysed after 1 week and 4 weeks of dosing. Given the small blood volumes obtained from the
Figure 25  Dose response study of “off target” growth factors in normal mice treated with ME1. 10 week old female BalbC mice were treated with ME1 IP on days 1,4,7 at the doses indicated. Blood samples were taken 24 hours after the last treatment and growth factors VEGF, PlGF-2, G-CSF, SDF1α, and SCF were assayed by ELISA. Symbols and bars, mean + SEM.
orbital sinus after 1 week in these mice that received ongoing treatment, the 1-week samples from each dose level were pooled for analysis. Lapatinib did not lead to a decrease in VEGF or G-CSF at lower doses, but did lead to increases in these growth factors at higher and more prolonged dosing (Figure 26). A dose of 100 mg/kg BID or above was not sustainable over 4 weeks, due to toxicity to the mice, manifesting as weight loss and inactivity, and the 50 mg/kg BID as well as 100 mg/kg and 120 mg/kg SID dose regimens were also not well tolerated over time. These doses were able to be given for 4 weeks, but were not suitable for longer term therapy experiments. These high doses are in the range of those that are associated with increased circulating VEGF and G-CSF in plasma. For therapeutic studies in the laboratory, a dose of 25 mg/kg BID has been utilized. Part of the reason for this surrounds results of CEP analysis (see below).

3.4.2 Effect of ME1 and lapatinib on CEPs in normal mice

The same dosing schedule was utilized to assess CEPs in normal non-tumour bearing BalbC mice treated with ME1 (administered every 3 days by IP injection) or Lapatinib (administered SID or BID by PO gavage). The flow cytometric assay for CEP quantitation has been applied to the study of many targeted agents, VDAs, and chemotherapy drugs in the laboratory. ME1 was associated with a dose-dependent suppression of CEPs that became significant at the 500 μg dose, after both 1 week and 4 weeks of treatment. The pattern of suppression became reversed at the higher 1000 μg dose level, resulting in what appears to be a U-shaped curve (Figure 27). Interestingly, this higher dose level
Figure 26  Assessment of mouse AR, VEGF, and G-CSF after long term treatment with the EGFR/HER2 RTKI lapatinib. Mice were treated by SID or BID PO gavage at the doses indicated. Blood samples were obtained by the orbital sinus after 1 week, then plasma was pooled for analysis. Growth factors were assayed by ELISA. Symbols and bars, mean + SEM.
Figure 27  Effect of anti-mouse EGFR antibody ME1 on viable CEPs in normal mice. Mice were treated with ME1 IP at the doses indicated every 3 days for 4 weeks. Blood was sampled in EDTA after 1 week and 4 weeks from the orbital sinus for vCEP enumeration using flow cytometry. Bars, mean ± SD.
was also associated with increases in proangiogenic cytokines, such as VEGF. After 4 weeks of lapatinib dosing, a similar U-shape curve was observed (Figure 28), with the largest CEP suppression noted with 25 mg/kg BID, but significant suppression was also noted with 10 mg/kg BID, as well as 50 mg/kg SID – a dose that also suppressed CEPs at the 1-week mark (Figure 28). Thus, it appears from these data that EGFR inhibitors, whether antibody or RTKI-based, may have the capacity to suppress CEPs in normal mice, and when used at an optimal dose, these drugs may manifest at least a portion of their anti-angiogenic activity through the suppression of systemic vasculogenesis. This response may be brought about through an increasing number of anti-cancer treatments, and can be mediated through growth factors such as VEGF or G-CSF.

3.4.3 Impact of chemotherapy on the ligand response induced by EGFR inhibition

Next we sought to investigate whether chemotherapy agents may influence the ligand elevations after treatment with EGFR monoclonal antibodies. We selected two agents to take forward into metronomic combination therapy experiments. Cyclophosphamide was tested at both MTD and LDM dose levels. Irinotecan (CPT11) was also tested, given that it has been used successfully at conventional doses in combination with cetuximab for colorectal cancer and has also been investigated as part of a metronomic regimen\textsuperscript{212}. Tests were conducted in normal BalbC mice. For the on-target EGFR ligands AR and EPR, neither MTD CTX, nor LDM CTX, nor CPT11 as single agent treatments were
Figure 28 Effect of EGFR/HER2 RTKI lapatinib on viable CEPs in normal mice. Mice were treated with lapatinib PO by gavage at the doses indicated either BID (top) or SID (bottom) for 4 weeks. The 100 mg/kg BID and 150 mg/kg SID doses could not be continued for 4 weeks due to toxicity. Blood was sampled in EDTA after 1 week and 4 weeks from the orbital sinus for vCEP enumeration using flow cytometry. 5 mice/group. Bars, mean ± SD.
associated with ligand increases (Figure 29). The magnitude of AR elevation produced by ME1 was attenuated by the presence of CTX but this was not observed with CPT11, and EPR levels were not influenced by either chemotherapeutic drug. Results for the off-target growth factors VEGF and SDF1α were more difficult to interpret, as no clear pattern emerged (Figure 29). Other recent work from the laboratory has investigated the impact of CTX MTD and LDM regimens on circulating growth factors more extensively, with sampling at 1, 7 and 21 days of dosing, revealing an increase in VEGF and G-CSF after 7 days with the MTD but not the LDM schedule of cyclophosphamide (Omar Lopez, personal communication).

3.5 Discussion

3.5.1 Inhibition of host EGFR

Unlike small molecule RTKIs, which possess less receptor selectivity and easier crossover between species, the monoclonal antibodies are often highly species specific, which is the case with cetuximab. Given the host-based nature of the on-target ligand elevations as a potential OBD biomarker, the acquisition of a host-specific anti-EGFR antibody was highly valuable, and experiments that evaluate other host responses, specifically off-target growth factors and CEPs. As mentioned in Chapter 3, the characteristic rash seen with EGFR inhibitors in clinical practice has also been a host response that is thought to mirror optimal drug use. While the BalbC mice treated with ME1 for our studies do not suffer from a rash, they develop a wavy hair phenotype after approximately 1-2 weeks
Figure 29  Evaluation of cyclophosphamide and irinotecan chemotherapy +/- ME1 on circulating growth factors in normal mice. Mice were treated with MTD (100mg/kg IP on days 1,3,5) or LDM (20mg/kg/day continuously) cyclophosphamide (CTX), or irinotecan. Blood samples were obtained by cardiac puncture after 1 week of therapy and assayed for mouse AR, EPR, VEGF, and SDF1α by ELISA.
of drug dosing (Figure 30). An abnormal haircoat phenotype has been described for EGFR knockout mice as well, and it developed early in mice treated with the 500 or 1000 μg doses, but was also apparent in mice treated with 250 μg daily for several weeks.

3.5.2 EGFR targeting and CEPs

Our experiments demonstrated a dose-dependent suppression of CEPs by the EGFR monoclonal antibody ME1 and the dual EGFR/HER2 RTKI lapatinib. The rationale for these experiments was to evaluate the impact that EGFR inhibitors have on VEGF and the potential to impact systemic vasculogenesis. We are encouraged by recently published results that come to similar conclusions to our own. One study demonstrated the contribution that TGFα signalling makes to vessel repair in stroke and how this pro-angiogenic effect involved the incorporation of CEPs into blood vessels in the infarct border zone 213. Another study documented a reduction in green fluorescent protein-labelled bone marrow cell incorporation into the syngeneic and negligible EGFR-expressing tumour model of B16 melanoma, upon gefitinib treatment 214.

An interesting question for future study regarding the impact that EGFR inhibitors have on CEP suppression is whether these drugs may be useful to block agents that cause a rapid CEP surge from the bone marrow to peripherally located tumours. A post-treatment increase in CEPs has been documented for VDAs 94, as well as certain chemotherapy drugs, such as paclitaxel 74, and this CEP response has been blunted by pre-treatment with an inhibitor of VEGFR-2.
Figure 30  Photograph of BalbC mouse after 2 weeks of treatment with ME1. Mouse on right received 500 μg ME1 IP every 3 days. Mouse on left received saline IP every 3 days. Hair phenotype may be due to EGFR blockade.
signalling. If the suppression of CEPs by EGFR-targeted drugs, even by an indirect mechanism, might be strong enough to blunt a chemotherapy-induced CEP response, this may shed light on a possible mechanism responsible for the chemosensitization that has been observed with drugs such as cetuximab and panitumumab.

3.5.3 The U-shaped curve

An interesting finding from these results is the suppression of CEPs, and possible reduction in circulating levels of VEGF, that reverses upon high dose treatment. This result may illustrate opposing outcomes of drug use that are dose-dependent. A drug, in this case ME1 or other EGFR inhibitors, may suppress angiogenesis at optimal doses, but promote angiogenesis through growth factor and CEP induction as the dose is increased, or perhaps given for longer time periods. Even for growth factors that were not suppressed at lower doses, such as G-CSF, an elevation became evident at the highest dose following treatment with all EGFR inhibitors studied: ME1, erlotinib, and lapatinib. In the case of ME1, a dose-dependent trend to increasing G-CSF was observed. As G-CSF has recently been tied to promoting the CEP response, this could represent an example of a switch to promotion of angiogenesis at higher drug dose. Therefore, the possibility of a U-shaped curve with targeted drug dosing, and the potential negative consequences of selecting a dose that is higher than the OBD, reiterates the importance of accurate identification of a dose that results in optimal (beneficial) activity. This might be difficult, as illustrated by
recent findings with angiogenesis inhibitors administered at the presumed OBD, resulting in the promotion of adverse outcomes, such as increased tumour invasion and/or metastasis \(^{76,77}\). Whether this effect occurs with certain drugs, doses, schedules, or tumour stages, is not known, but is an area of investigation. Whether EGFR targeted drugs, utilized at the OBD, may also affect angiogenesis, invasion, or metastasis in a negative way, remains to be determined. The results from this chapter illustrate characteristics that may be shared between EGFR and VEGFR inhibitors regarding effects on circulating cytokines and CEPs.
Chapter 4

Combining Targeted EGFR Monoclonal Antibody Treatment with Metronomic Cyclophosphamide Chemotherapy†

4.1 Abstract

The objective of this study was to evaluate the anti-EGFR humanized monoclonal antibody nimotuzumab in combination with metronomic cyclophosphamide in preclinical models of advanced colorectal cancer and metastatic triple negative breast cancer. Nimotuzumab has demonstrated clinical activity without causing severe side-effects including skin rash and hypomagnesaemia. Low dose chemotherapy regimens using cyclophosphamide are also well tolerated and suitable for long term therapy. Experiments with preclinical models of colorectal cancer are ongoing, and current results are summarized in Appendix 3. For the advanced breast cancer preclinical model, increasingly metastatic variants of the MDA-MB-231 human breast cancer cell line were selected through passage in vivo, resulting in a high EGFR-expressing line which was metastatic to many organs, including lymph nodes, lung, and brain. The treatment with nimotuzumab, metronomic cyclophosphamide, or the combination, was well tolerated. Combination therapy resulted in significant primary tumour growth delay in the metastatic variant, compared to the parental cell line. Similar results were obtained using cetuximab in the primary tumour growth delay model. Combination treatment also resulted in a significant survival

† Portions of this chapter (advanced breast cancer model) have been submitted for publication by Mutsaers AJ et al.
advantage over cyclophosphamide alone in the advanced metastatic model, and decreased the size of tumour recurrences and lymph node metastasis. At the completion of this experiment, two cell lines were established from mouse lung metastases and these cells retained EGFR expression. The combination of metronomic cyclophosphamide and EGFR-targeting monoclonal antibody was efficacious and well tolerated in this preclinical model. This treatment approach should be considered for triple negative breast cancer patients in future clinical trials.

4.2 Introduction
4.2.1 Metronomic chemotherapy and EGFR inhibition for colorectal cancer

Our interest in metronomic chemotherapy regimens has been applied to numerous tumour types, and one persistent lesson from these studies has been the increased potency of such protocols when used in combination with targeted anti-angiogenic drugs. Therefore, when searching for appropriate tumour types to investigate metronomic chemotherapy in combination with EGFR antibody therapy, a natural choice was colorectal cancer, as treatment of this tumour is an indicated use for cetuximab and panitumumab in combination with irinotecan/CPT11. The choice of cyclophosphamide for these experiments might be counter intuitive, given that it is not a recognized drug for treatment of this disease, unlike CPT11. However, since the original work by Browder et al demonstrated the endothelial-specific action of metronomic cyclophosphamide, activity may not depend on drug sensitivity in the tumour cell population.
For this reason, we continue to investigate metronomic CTX administered daily through the drinking water in a variety of preclinical models, although the requirement for tumour-cell sensitivity with metronomic protocols remains unresolved. Recent literature has reported metronomic approaches for treatment of HT29 colorectal cancer subcutaneous xenografts with CPT11 in combination with the VEGFR-2 RTKI semaxinib, as well as paclitaxel in combination with cetuximab. Preclinical models of colorectal cancer already used extensively in this thesis have included two colorectal cancer cell lines, GEO, and HT29. The GEO cell line has inherent sensitivity to treatment with EGFR inhibitors, including cetuximab and nimotuzumab. Subcutaneous GEO xenografts showed an inherent resistance to CTX, but better responses were seen with the combination of CTX and cetuximab over mice treated with cetuximab alone (see Appendix 3 for original, unpublished colorectal xenograft data). Ectopic xenograft models may provide information about drug efficacy, but it is important to develop more representative models of primary tumour growth, and of advanced metastatic disease, where new cancer therapies receive their first clinical investigation. We have therefore investigated HT29 grown IP, as reported in Chapter 2 and in previous studies, but also after intra-splenic injection, to facilitate metastasis to the liver and other organs (Appendix 3). To permit tracking of tumour burden in these studies, we utilized the HT29 cells that had been transfected with β-hcg, but also luciferase, to permit tumour localization with a xenogen camera. This model continues to be refined, and treatment with CTX and cetuximab did not reveal a reduced tumour burden or survival
advantage (Appendix 3). We have also investigated the combination of cetuximab and host-directed ME1 in this model, to assess the impact of both host and tumour EGFR blockade, with similar negative results (Appendix 3). The pursuit of efficacious metronomic therapy combinations and the development of orthotopic and metastatic preclinical models of colorectal cancer are ongoing, with recent extension to intracecal tumour implantation (Christina Hackl, personal communication). It will be interesting to see whether positive results with combination therapeutic approaches can be achieved in these challenging, but more clinically relevant orthotopic and metastatic models of this disease.

Another cancer type where we have been applying models of advanced metastasis has been breast cancer\textsuperscript{217-219}. This disease has shown response to metronomic scheduling of cyclophosphamide (a drug used extensively for breast cancer treatment), and there has been recent interest in EGFR targeting in subtypes of this cancer\textsuperscript{220}. We therefore sought to investigate metronomic CTX with EGFR monoclonal antibodies in a preclinical model of advanced breast cancer. The results of this effort constitute the remainder of this chapter.

\textbf{4.2.2 Metronomic cyclophosphamide and EGFR inhibition for breast cancer}

Low dose chemotherapy administered at regular intervals without prolonged breaks, (also referred to as metronomic chemotherapy) is a treatment approach that is being investigated in a number of cancer types, including breast cancer. A clinical trial of low dose, metronomic chemotherapy with daily oral cyclophosphamide and twice weekly oral methotrexate for treatment of
metastatic breast cancer showed an overall clinical benefit in 20 (31.7%) of 63 patients. Overall clinical benefit was defined as the number of patients that experienced a complete remission, partial remission, or stable disease for greater than 24 weeks. This chemotherapy regimen was minimally toxic and efficacious in a patient population that had been heavily pre-treated. The mechanism for efficacy with metronomic regimens has largely been attributed to an anti-angiogenic effect, although other mechanisms, such as a direct anti-tumour cell and/or immunostimulatory effects may also be involved.

Optimal benefit for metronomic scheduling of chemotherapy is thought to occur when these agents are used in combination with other targeted antiangiogenic and/or relevant targeted antitumour drugs. Accordingly, the cyclophosphamide/methotrexate or a cyclophosphamide/capecitabine regimen have also been evaluated in combination with 2 targeted agents that have been approved for breast cancer therapy, namely, trastuzumab (Herceptin®) the anti-HER2/erbB2 monoclonal antibody and bevacizumab (Avastin®), with overall clinical benefit of 46% and 64-68% respectively. The addition of another anti-angiogenic compound, thalidomide, to the cyclophosphamide/methotrexate regimen, did not improve results over metronomic chemotherapy alone.

Triple-negative (estrogen receptor, progesterone receptor, ErbB2/HER2-negative) breast cancers are tumours with an aggressive behaviour and limited therapeutic options, due to the lack of applicability of either endocrine-based treatment, such as tamoxifen and letrozole, or anti-HER2 therapy with
trastuzumab\textsuperscript{225,226}. The epidermal growth factor receptor (EGFR) pathway may be a relevant target for the treatment of certain triple negative breast cancers\textsuperscript{220}, and there are clinical trials investigating EGFR inhibition for this disease, alone or in combination with chemotherapy, such as carboplatin (http://www.clinicaltrials.gov/ct/show/NCT00232505) or paclitaxel, delivered in a conventional MTD design, but not in a continuous, metronomic fashion. Several EGFR inhibitors are approved or are being evaluated in clinical trials for a variety of cancers. Nimotuzumab is a humanized monoclonal antibody targeting EGFR with an affinity that is lower than currently approved agents, such as cetuximab and panitumumab\textsuperscript{227-233}. The result is a minimal toxicity profile, including reduction in incidence of the characteristic acneiform rash that is common with other EGFR targeted agents\textsuperscript{233-236}. An EGFR inhibitor with such an attractive toxicity profile, if efficacious, is well suited for long term dosing. Nimotuzumab is an attractive partner for combination therapy with minimally toxic metronomic chemotherapy regimens that, in contrast to MTD protocols, are also meant to be administered for prolonged periods of time. The objective of these experiments was to evaluate metronomic cyclophosphamide chemotherapy in combination with nimotuzumab in a preclinical model of highly aggressive and metastatic triple negative breast cancer. To accomplish this, increasingly metastatic variants of the er/pr/HER2-negative MDA-MB-231 human breast cancer cell line were selected through passage \textit{in vivo}\textsuperscript{218;237;238}, resulting in a line with increased EGFR expression, termed 164/8-1B.
4.3 Methods

4.3.1 Drugs and Schedule

Cyclophosphamide (Baxter Oncology GmbH, Mississauga, ON, Canada) was purchased from the institutional pharmacy; it was reconstituted as per instructions of the manufacturer to a stock concentration of 20mg/ml and administered via the drinking water at a dose of 20mg/kg/d based on an estimated daily consumption of 3ml for a 20g mouse, as previously described by Man et al, 2002 \textsuperscript{183}. Nimotuzumab was supplied by YM Biosciences (Mississauga, ON, Canada); it was administered at of dose of 0.5mg/mouse by intraperitoneal injection twice weekly. Saline was used as a control in both the drinking water and IP injection treatment groups.

4.3.2 Cells and culture conditions

MDA-MB-231 cells were originally a generous gift from Dr. Jeff Lamontt. All metastatic variants from this parental cell line were generated according to the method described by Munoz et al. 2006 \textsuperscript{218}. The metastatic variants 8-1B and 1-3B were derived from the LM2-4 variant in a similar fashion. All cell lines were cultured in RPMI 1640 supplemented with 5% fetal bovine serum (Invitrogen Life Technologies Inc., Burlington Ontario, Canada). Cells were grown in monolayer culture to 75-90% confluence, detached, made into single cell suspensions using 0.05% trypsin-EDTA and washed twice in FBS-free medium prior to EGFR analysis or injection \textit{in vivo}. Viable cells were counted using Trypan Blue prior to injection.
4.3.3 Assessment of surface EGFR expression

Cells were prepared as described above and examined by flow cytometry using a BD Caliber and PE-conjugated anti-human EGFR primary antibody and isotype control from BD Pharmingen. One million cells were placed in each of 3 tubes (for unstained, isotype control, and EGFR stained cells, respectively). Cells were centrifuged at 16000g at 4 °C for 5 minutes, then resuspended in Hank’s balanced salt solution in 2% FCS (HBSS-2%-FCS). 20 μl of antibody (or HBSS-2%-FCS) was added to each tube and incubated for 30 minutes on ice, with gentle agitation twice during the incubation period. Tubes were washed twice with HBSS-2%-FCS, then 0.6 ml of HBSS-2%-FCS containing 1 μg/ml 7AAD was added. Samples were filtered through a 5 ml filter top Falcon tube and stored protected from light until analysis. Results were displayed by mean fluorescence intensity.

4.3.4 In Vivo experiments

Two million cells were orthotopically transplanted into the mammary fat pad of female CB17 SCID mice (Charles River Canada). For primary tumour growth delay experiments, treatment began when tumours reached approximately 200mm³. Tumour volume was calculated using the formula length*width²/2. For the metastatic model, tumours were removed after reaching approximately 500mm³, and treatment began 14 days later. n=5 mice/group for primary tumour experiments and n=10 mice/group for the metastatic model.
Each experiment was performed in duplicate. All experimental protocols were approved by the institutional animal care and use committee.

4.3.5 Statistical analysis

Results are reported as mean ± SEM. Differences in survival were assessed by the method of Kaplan & Meier Logrank test and chi square analysis using Graph Pad Prism version 4 software (San Diego, CA). A p value of < 0.05 was considered significant.

4.4 Results

4.4.1 Selection of a highly metastatic EGFR-expressing variant of the human MDA-MB-231 breast cancer cell line

As part of an ongoing effort in our laboratory to generate preclinical models of advanced metastatic disease with which to investigate experimental therapeutic combinations, we have been selecting metastatic variants of the MDA-MB-231 human breast cancer cell line, as described in detail in Munoz et al, 2006 and subsequently by Francia et al. The MDA-MB-231 cell line is estrogen receptor, progesterone receptor, and ErbB2/HER2-negative, and has been utilized previously, along with its variant LM/2-4, by our laboratory for evaluation of metronomic treatment strategies. The 1-3B and 8-1B variants were cultivated from a brain metastasis that arose out of an LM/2-4 orthotopic xenograft. After establishment as a cell line and repeated inoculation into the mammary fat pad with subsequent resection, this 8-1B variant...
reproducibly established lymph node, lung and brain metastases in mice (Figure 31).

Cell lines from the parental MDA-MB-231, as well as variants LM2-4, 1-3B, and 8-1B were analyzed for cell surface EGFR expression by flow cytometry. Each cell line had detectable EGFR expression when compared to the isotype negative control antibody, and EGFR expression was progressively greater in the metastatic variants, with the 8-1B line having the highest expression (Figure 31). The 8-1B and 1-3B variants were both selected from the LM2-4 cell line, which itself originated out of selection from the parental MDA-MB-231. Due to its higher EGFR content and highly metastatic phenotype, the 8-1B cell line was chosen for subsequent in vivo experiments.

4.4.2 Effects of single agent cyclophosphamide or nimotuzumab versus the combination on primary tumour growth delay in parental and 164/8-1B orthotopic xenografts

We first tested this treatment regimen on orthotopically transplanted tumours to investigate this approach in a more conventional model. Tumours were injected into the right inguinal mammary fat pad and treatment began once tumours reached a size of approximately 200mm³. In contrast to the parental cell line, the 8-1B tumours grew faster, and were more responsive to treatment (Figure 32). Because of this difference in growth rate, treatment began on day 32 after tumour implantation for the parental cell line versus day 12 for the 8-1B variant. The combination of cyclophosphamide and nimotuzumab was
**Figure 31** Cell surface EGFR expression from MDA-MB-231 and metastatic variants, plus histology of brain and myocardial metastases from the 164/8-1B variant. Cell lines were grown in monolayer culture and evaluated for surface EGFR expression by flow cytometry. Sections of brain and heart were obtained from 3 different mice at the time of sacrifice. Mice had 164/8-1B cells implanted into the mammary fat pad, then resected after 12 days. Slides stained with hematoxylin and eosin.
Figure 32  Treatment of MDA-MB-231 and 164/8-1B primary tumours with metronomic cyclophosphamide and nimotuzumab or cetuximab. Cells were inoculated into the right mammary fat pad. Treatment began when tumours reached 200 mm³. 5 mice/group. Symbols and bars, mean + SEM.
moderately effective in delaying tumour growth in the parental cell line, particularly at later stages of tumour growth. There was also some benefit with cyclophosphamide alone but there was no activity with nimotuzumab alone. In contrast, there was a more robust effect with single agent therapy with cyclophosphamide as well as nimotuzumab in the more aggressive 8-1B variant, and combination therapy was very effective in delaying tumour growth in this model (Figure 32). In addition to nimotuzumab, we tested cetuximab in the 8-1B primary growth delay model. The results with cetuximab were very similar to those produced with nimotuzumab (Figure 32).

4.4.3 Effects of single agent cyclophosphamide or nimotuzumab versus the combination on survival of mice with advanced metastatic disease

The next experiments involved treatment of advanced metastasis. The 8-1B variant was injected orthotopically and this time grown to a size of approximately 500mm$^3$. At that point, (approximately 3 weeks) the tumours were surgically removed from the mammary fat pad, and treatment was delayed for a further 2 weeks, to allow growth of metastatic foci in multiple organ sites. A nimotuzumab dose of 0.5 mg twice weekly did not have any significant effect on the mice, although 1 mouse was a long term survivor, with no evidence of metastatic disease 10 months after the tumour was originally implanted, which was 5 months after therapy was stopped. The metronomic cyclophosphamide monotherapy group showed prolonged survival, with a median survival time of 91 days, compared to 67.5 days for the control group (Figure 33, Logrank p=0.024).
However, the 116.5 day median survival for the combination group was significantly longer than cyclophosphamide monotherapy (Figure 33, Logrank p=0.0066). Body weight was utilized as an indicator of toxicity of the treatment protocols. Long term treatment with the combination of cyclophosphamide and nimotuzumab did not result in toxicity at any time during the 112 day treatment period (Figure 33).

4.4.4 Effects of treatment on tumour recurrence and metastasis

In the advanced metastasis experiments, where primary tumours were surgically removed, regrowth in the right inguinal mammary fat pad and/or invasion into the adjacent abdominal musculature occurred at variable times, but with similar frequency across treatment groups (4-6 mice out of 10 per group, Figure 34). However, the resulting tumours were smaller in mice receiving combination treatment (Figure 34). Of 40 mice in this experiment, 2 mice were sacrificed specifically due to size of tumour recurrence (beyond 1.7cm³), and both of these had received cyclophosphamide alone. Lymph node enlargement was also observed in 4-6 mice of 10 in each treatment group, with the exception of those that received combination therapy, where no gross evidence of lymphadenomegaly in any mouse was observed (Figure 34).

4.4.5 EGFR target expression in lung metastases

As part of an ongoing attempt to investigate possible causes of drug resistance, as well as obtain and characterize more aggressive metastatic
Figure 33  Survival curve for the 164/8-1B advanced metastasis model treated with metronomic cyclophosphamide and nimotuzumab. Cells were orthotopically inoculated into the right mammary fat pad. Tumours were resected when they reached 500 mm3. After 14 days treatment was initiated. Body weight was monitored as an indicator of toxicity. n = 10 mice/group.
Figure 34  Incidence and size of tumour recurrence and incidence of lymphadenomegaly in the advanced metastatic model treated with metronomic cyclophosphamide and nimotuzumab. Two mice required sacrifice due to size of tumour regrowth (both in single agent cyclophosphamide group). No lymphadenomegaly was observed in mice treated with the combination therapy. Symbols and bars, mean + SEM.
tumour variants, gross lung metastases from 2 mice that had received combination therapy were harvested at the time of sacrifice (Figure 35). These tissues were adapted to cell culture, resulting in the generation of 2 individual tumour cell lines, referred to as 1-3L and 1-5L. To characterize whether the resistance to combination therapy may have been associated with a loss of EGFR expression, the cell lines were tested for cell surface EGFR content by flow cytometry, as described earlier. Both cell lines showed surface EGFR expression, suggesting that resistance to treatment was not associated with loss of target expression (Figure 35). However, while EGFR content was similar between 1-3L and the original 8-1B cell line, surface EGFR expression in the 1-5L line was reduced by approximately 47%.

4.5 Discussion

4.5.1 Preclinical models of advanced, metastatic breast cancer

Our initial goal has been to develop preclinical models of advanced breast cancer, to permit evaluation of novel treatment approaches in a setting that more closely approximates what occurs when a specific therapy enters preliminary (phase I and II) clinical trial evaluation. In addition to our previous studies with MDA-MB-231 and its variant LM2-4, these results with the second generation variant 8-1B more closely resemble a high EGFR expressing aggressive triple negative breast cancer. Triple negative breast cancers more frequently metastasize to visceral organs, including the CNS, and this cell line also shows aggressive behaviour. We have also engineered some of the
Figure 35  Surface EGFR expression in 2 lung metastatic variants cultivated from mice in the advanced metastatic model. A grossly visible lung metastasis from 2 mice from the combination therapy group was adapted to cell culture, and tested along with the parental 164/8-1B cell line for EGFR expression by flow cytometry.
earlier generation cell lines to express HER2 and successfully studied their response to metronomic chemotherapy regimens paired with the targeted agent trastuzumab\textsuperscript{237:240-242}. Ongoing work involved characterizing other breast cancer cell lines for these studies, and the MDA-MB-468 line, which expresses a very high level of EGFR (Figure 36), appears promising in this regard.

4.5.2 Metronomic chemotherapy choices for breast cancer treatment

Our results expand a growing list of potential targeted therapies to be used with metronomic cyclophosphamide containing regimens for the treatment of metastatic breast cancer, to include EGFR inhibition, where this pathway may be relevant to tumour growth and/or metastasis. Previous clinical trials in breast cancer have shown beneficial results with low dose, continuous cyclophosphamide-containing protocols, alone and in combination with the targeted drugs trastuzumab or bevacizumab \textsuperscript{107:243}. The encouraging results obtained in these clinical trials, along with the interest in EGFR targeting in triple negative breast cancer, and the data presented herein with metronomic cyclophosphamide and nimotuzumab, suggest this approach should be evaluated clinically. Based on previous reports, it is possible that doublet chemotherapy protocols could improve upon the antitumour response. In addition to 50mg daily oral cyclophosphamide treatment, previous metronomic breast cancer trials have also included methotrexate, given either at 2.5mg orally twice daily on day 1 and 2 of a weekly cycle, or 1mg/kg IV every 14 days. There
Figure 36 High EGFR expression in the MDA-MB-468 cell line. Ongoing work has involved characterizing human breast cancer cell lines for testing in advanced metastatic preclinical models. The MDA-MB-468 cell line has much higher surface EGFR expression than 164/8-1B. A variant of the MDA-MB-468 cell line has been cultivated from a parental tumour growing in vivo, for future passage in orthotopic xenografts (MDA-MB-468T).
remains uncertainty about the optimal dose and schedule for the drugs used in metronomic chemotherapy protocols.

Another chemotherapeutic that has been administered metronomically, and shown promise on its own and in combination with cyclophosphamide, is the 5-fluorouracil oral prodrug Uracil+Tegafur, or UFT. This drug is approved outside North America for breast cancer treatment, and has shown promising results when given in the adjuvant setting, on a 2-year daily non-toxic, metronomic-like continuous schedule \(244\). In previous preclinical studies of breast cancer xenografts, conducted by our laboratory and others, the combination of UFT and cyclophosphamide has been particularly efficacious, and one possible reason to explain the benefits of this combination may be inhibition of dihydropyrimidine dehydrogenase activity by the cyclophosphamide, resulting in augmented UFT efficacy, or enhancement of thymidylate synthetase activity – the target of 5-fluorouracil \(245\). Given these observations, cyclophosphamide and UFT, in combination with an EGFR inhibitor, could have better efficacy in triple negative breast cancers than an EGFR inhibitor used with single agent chemotherapy. Dellapasqua et al recently reported encouraging results in a phase II metastatic breast cancer trial of metronomic cyclophosphamide plus concurrent metronomic capecitabine given daily with bevacizumab every 2 weeks \(107\). This protocol is being evaluated in a phase III trial in Europe, against the approved weekly paclitaxel plus bevacizumab regimen (http://sakk.ch/en/).
4.5.3 EGFR drug choices, toxicity, and resistance

Another area for optimization is how to choose drugs for blockade of EGFR. Nimotuzumab improved treatment response in our 8-1B preclinical xenograft model in combination with metronomic cyclophosphamide, and given the lower affinity of this antibody for EGFR, it has been shown to preferentially bind high expressing EGFR tumours, and spare normal tissues. This drug should work optimally for breast cancer patients with high EGFR-expressing tumours, but EGFR expression has not predicted benefit with this class of drugs in other tumour types, such as colorectal cancer. It is not known whether EGFR expression will define a population of breast cancer patients that will respond to this class of drugs. The low toxicity profile of nimotuzumab may make combination with other EGFR drugs – either other antibodies or small molecule inhibitors, more beneficial without a concomitant increase in toxicities, such as skin rash, and there is evidence to support these types of combination approaches. We tested nimotuzumab and cetuximab in combination in our model, and did not observe an increase in efficacy over nimotuzumab or cetuximab alone (Figure 37). The potential overlap of epitopes between these two antibodies may be one explanation for lack of improvement over single antibody therapy. In addition to surface levels of the target EGFR, the potential expression of nuclear EGFR, and its specific role in our preclinical model was not investigated, but may contribute to tumour growth, might be inhibited with small molecule drugs versus antibodies. Also, it is expected that the lack of binding to EGFR on mouse tissues contributes to a lack of
Figure 37 Nimotuzumab and cetuximab used in combination for the treatment of 164/8-1B orthotopic xenografts. Tumour and treatment conditions were identical to those from Figure 32.
observed side effects when anti-human receptor antibody drugs are tested preclinically in mice. Fortunately, with nimotuzumab, there are clinical data demonstrating its tolerability in patients, and its favourable toxicity profile is a major reason it was chosen for these studies with long term, low dose cyclophosphamide chemotherapy. Lessons from the combination of cetuximab, bevacizumab and chemotherapy for colorectal cancer suggest that favourable toxicity profiles and benefit from preclinical modeling, does not necessarily bear out in clinical trials\textsuperscript{254}.

We observed that tumours harvested from the lungs of mice that became resistant to combination therapy retained surface EGFR expression. This may represent a form of acquired evasive drug resistance, where tumour cells utilize alternative pathways to permit growth in the presence of drug blockade of a particular target. The demonstration of IGF-1 receptor pathways as a resistance mechanism to HER2 targeting with trastuzumab is one example\textsuperscript{255-257}. Other resistance mechanisms may occur, such as host adaptive responses in the metastatic microenvironment\textsuperscript{258}. The nature of resistance to metronomic chemotherapy alone or in combination is an area of ongoing study\textsuperscript{259}. In the meantime, we suggest that the combination of encouraging metronomic cyclophosphamide-containing chemotherapy protocols with EGFR targeted therapies, such as nimotuzumab, should be considered for clinical evaluation in triple negative breast cancer patients. Assessment of tumour EGFR expression in these patients should also be undertaken, to evaluate its potential as a biomarker of patient selection. Similarly, combination of nimotuzumab with
metronomic chemotherapy regimens developed and found to be effective for head and neck cancers – which generally express very high levels of EGFR, would seem worthy of evaluation\textsuperscript{231,232}. 
Chapter 5

Summary and Discussion

5.1 Summary of the principal findings of this thesis

The major aim of this thesis was to investigate on-target and off-target growth factor and CEP changes in circulation with EGFR antibody drug treatment, then develop and test preclinical models of EGFR targeted antibody drug combinations with metronomic chemotherapy. This section summarizes the experimental findings as they pertain to the hypotheses outlined in the introductory section of this thesis.

Regarding on-target ligand elevations: Among the various EGFR ligands, TGFα appears to have the greatest potential for use as a biomarker of optimal cetuximab dose. The lower affinity antibody nimotuzumab did not cause TGFα elevation at comparable doses. However, it is possible that nimotuzumab may be able to displace lower affinity ligands, such as AR or EPR. Based on results in normal mice and EGFR-negative colorectal cancer patients, the origin of this ligand elevation appears to be host-tissue based, as has been demonstrated with an increase in circulating growth factors upon VEGFR-2 blockade. Finally, EGFR ligand elevations were confirmed to be unaffected by treatment with EGFR small molecule RTKIs in all models we tested.

Regarding off-target growth factor changes: While levels of the angiogenic cytokine VEGF are reduced with EGFR directed therapy, higher drug doses with a number of EGFR inhibitors result in increased circulating VEGF, as
well as G-CSF, but not SDF1 or SCF. These findings differ from those previously described for VEGF RTKIs, such as sunitinib and sorafenib (where all 4 growth factors were increased). The elevations in circulating plasma VEGF and G-CSF could have implications for tumour biology, such as contributing to progression, metastasis or resistance, as well as promoting recruitment of CEPs.

Regarding EGFR inhibition and CEPs: CEPs were reduced in a dose dependent manner by inhibitors of EGFR using an antibody (ME1) as well as the RTKI lapatinib. Interestingly, this result was not retained at higher drug doses, and these higher doses also resulted in a stimulatory effect on circulating levels of VEGF and G-CSF – two growth factors known to play a role in CEP mobilization.

Regarding EGFR monoclonal antibody treatment combined with metronomic chemotherapy in preclinical cancer models: Both nimotuzumab and cetuximab caused growth delay of the primary tumour in a preclinical model of triple-negative breast cancer, when either drug was used alone, and in combination with metronomic cyclophosphamide. The combination of nimotuzumab and cyclophosphamide also prolonged survival in a model of advanced metastatic disease, reduced the size of primary tumour regrowth, and decreased lymph node enlargement. Given its low toxicity profile, nimotuzumab may be a suitable partner for long term dosing in a clinical trial investigating EGFR inhibition in combination with metronomic chemotherapy, including cyclophosphamide, for triple negative breast cancer patients.
5.2 Discussion

5.2.1 EGFR antibody affinity vs. efficacy

One advantage in these projects has been the availability of EGFR inhibitors from both drug classes (i.e. monoclonal antibodies and RTKIs), but also antibodies of differing affinity for EGFR, as well as preferential binding of mouse vs. human receptor. There are many EGFR ligands, and in this thesis TGF\(\alpha\) emerged as a possible pharmacodynamic biomarker for the EGFR antibody cetuximab. Preclinical and clinical study of nimotuzumab did not support extension of the TGF\(\alpha\) results to this drug, and the reasons for this may be multiple and interrelated. Given the high affinity of TGF\(\alpha\) for EGFR, it appears possible that it is not able to be displaced by nimotuzumab. Also, since the predominant source of TGF\(\alpha\) with cetuximab treatment is most likely from host and not tumour tissues, it is not surprising to see a lack of circulating TGF\(\alpha\) elevation with nimotuzumab, as it also fails to produce other characteristic EGFR-related host tissue effects, namely the characteristic acneiform rash\(^{236;246}\). By contrast, the higher affinity and characteristic side effect profile associated with other EGFR monoclonal antibodies, such as panitumumab, make it another possible candidate drug to increase circulating TGF\(\alpha\) in patients.

Lack of host tissue-related increases in plasma levels of growth factors does not preclude a drug such as nimotuzumab from having anti-tumour activity. Some investigations claim that this drug achieves its desirable therapeutic index by selective binding to clusters of EGFR expressed at high levels at the cell surface\(^ {260}\). This drug is effective in preclinical models of EGFR-dependent
tumours, such as the cell lines A431 and GEO \(^{261}\), and we have observed its anti-tumour effects in our preclinical model of metastatic breast cancer. Successful use of nimotuzumab requires patients whose cancers are driven by EGFR signalling, perhaps induced by lower affinity ligands such as AR and EPR, and/or express very high cell surface receptor levels. EGFR expression is not a determinant of activity of cetuximab when treating colorectal cancer, and there is need of biomarkers that predict patient response. If an appropriate patient population can be defined, then long term treatment with an EGFR-targeted drug that does not result in cutaneous rash would be desirable, particularly when it is used in combination with metronomic chemotherapy, or other targeted agents, especially those designed to be administered for prolonged periods.

### 5.2.2 Blockade of host vs. tumour EGFR

The lack of host response to nimotuzumab is important. Our ability to utilize the anti-mouse EGFR antibody ME1 confirmed host responses brought about by EGFR blockade, but prior to clinical evaluation and approval of the EGFR antibodies cetuximab and panitumumab, preclinical efficacy models were based upon blockade of human EGFR expressed by transplanted human tumour xenografts. There was no prior awareness of host responses in preclinical models, as these agents did not bind mouse EGFR. As these two antibody drugs have met with some clinical success, this has not been much of an issue; however with the recent reports of two negative (and in fact, worse outcome) phase III clinical trial reports when panitumumab (PACCE) as well as cetuximab
CAIRO2 were combined with bevacizumab plus FOLFOX chemotherapy for treatment of colorectal cancer\textsuperscript{262}, the consequences of concurrent blocking of host cell EGFR and VEGF(R) should be evaluated more closely in preclinical models. Like cetuximab and panitumumab, bevacizumab is also an antibody that only inhibits the human protein, and does not bind host VEGF in preclinical models.
Chapter 6
Further Studies and Future Directions

6.1 Does the ligand OBD biomarker apply to still other targeted drugs?

There are many interesting and as yet unresolved questions that have arisen directly or indirectly from the studies undertaken for this thesis. One question is that which served as the basis for the project, namely whether other families of drugs, particularly antibodies, lead to elevated levels of circulating ligands in a dose dependent fashion? There are other drug targets within the HER family, including HER2, which is a receptor that is effectively neutralized by the monoclonal antibody trastuzumab. Even though there is no ligand for HER2, this receptor does form a dimer with EGFR that results in efficient down stream signal transduction. Another monoclonal antibody, pertuzumab, has been designed to specifically interfere with this dimerization, as well as those formed by HER2 with other HER family members. We treated MDA-MB-231 cells that had been transfected to express HER2 (called H2N) with trastuzumab and pertuzumab, as well as cetuximab, and compared TGF\(\alpha\) levels in conditioned media with that of the parental cell line. Treatment of the H2N cells resulted in a higher level of TGF\(\alpha\) in conditioned media compared to the parental cell line (H2N cells were shown to express higher phosphorylated EGFR in a recent study) and both trastuzumab and pertuzumab resulted in mild TGF\(\alpha\) elevations, particularly in the H2N cell line that expresses HER2 (Figure 38). In a recent study, pertuzumab was shown to increase EGFR homodimers through
Figure 38 Changes in TGFα in a HER2⁺ cell line treated with cetuximab, pertuzumab, or trastuzumab in vitro. Parental MDA-MB-231 cells and HER2-transfected variant H2N were incubated with each of the 3 antibodies, and conditioned media was assayed for TGFα by ELISA after 24 hours. Bars, mean + SD.
dissolution of EGFR-HER2 heterodimers, which may make more EGFR target available for ligand (or antibody drug) binding \textsuperscript{264}. TGF\textsubscript{α} levels were over 10-fold higher with cetuximab treatment compared to levels produced with pertuzumab and trastuzumab.

We also investigated antibodies directed at the human PDGFR\textsubscript{α} (IMC-3G3) and mouse PDGFR\textsubscript{β} (IMC-2C5). The former was tested in vitro with monolayer cell cultures of the human glioma cell line U118 which expresses PDGFR\textsubscript{α} \textsuperscript{265} and the human breast carcinoma cell line HS578T, also considered PDGFR\textsubscript{α\textsuperscript{+}}. Each of these cell lines produced dose dependent increases in human PDGF-AA ligand in conditioned media, and similar to studies within the VEGFR and EGFR systems, the results were not affected by viable cell count or proliferation indices (Figure 39). The 2C5 antibody was administered to normal BalbC mice 3 times over a week and plasma evaluated for on-target and off-target growth factors. Unlike other growth factors, the PDGF levels had wide variability between mice, which made drawing conclusions difficult with small numbers. However, at doses of 250 \(\mu\)g and above, this antibody may lead to increased PDGF-BB ligand, and decrease off target ligands VEGF, G-CSF, and SDF\textsubscript{α} (Figure 40). Further studies are required to confirm these results. The small molecule inhibitors of PDGFR\textsubscript{β} sunitinib and sorafenib, have also been evaluated for their ability to cause changes in plasma levels of these growth factors, also with similar wide variability in results \textsuperscript{45}.

The IGF-1 receptor represents an attractive target in oncology, and inhibitors have proceeded successfully into advanced clinical development \textsuperscript{266}. 

Figure 39  Elevated PDGF-AA in conditioned media after treatment with the anti-PDGFRα antibody IMC-3G3 in vitro. The U118 glioma and HS578T breast carcinoma cell lines were incubated with IMC-3G3 for 24 hours and PDGF-AA was assayed in conditioned media by ELISA. MTS assay on U118 cells did not show a significant effect from drug treatment. Symbols and bars, mean ± SD.
Figure 40  Dose response with anti-PDGFRβ antibody IMC-2C5 in vivo. 10 week old BalbC mice were treated with a single dose of antibody IP and a blood sample was obtained 24 hours later by cardiac puncture. The growth factors PDGF-BB, VEGF, G-CSF, and SDF1α were assayed from plasma by ELISA. 5 mice/group. Bars, mean ± SEM.
Two separate trials with IGF-1R monoclonal antibodies have recently
demonstrated increased circulating IGF-1 in patient serum\textsuperscript{267,268} (Appendix 4). Similar to other receptor tyrosine kinases, internalization following antibody engagement and subsequent receptor downregulation appears to be common with IGF-1R\textsuperscript{269-271}. A precedent for ligand elevation following inhibition of IGF-1R dependent pathways has been reported in children with congenital IGF-1R mutations, who demonstrated elevated levels of circulating IGF-1\textsuperscript{272}. In conclusion, it appears that the paradigm of elevated ligand upon extracellular receptor neutralization may be applicable for a number of kinases.

6.2 Do VEGFR-2 targeted drugs alter EGFR ligands?

The flow of information regarding applicability of results with VEGFR-targeted agents to those targeting EGFR in this thesis has gone in one direction only. If cross talk is prevalent between these signalling systems, it raises the question of whether EGFR ligands might be affected by drugs that target VEGFR-2. Preliminary evidence to support a dose-dependent increase in AR was obtained with a drug that hasn’t been utilized in experiments for this thesis, namely pazopanib (Figure 41), a RTKI that targets VEGFR-2. The minimum SID and BID doses that caused increases in AR (150 mg/kg and 25mg/kg respectively) were the same doses that were independently chosen for long term therapy experiments within the laboratory\textsuperscript{259}. Pazopanib, as well as other RTKI drugs, such as sunitinib and sorafenib, are multi-targeting, and it is not possible to exclude some drug binding directly to the EGFR kinase domain, particularly at
Figure 41  Dose dependent increase in circulating AR upon treatment with the VEGFR-2 RTKI pazopanib. 10 week old BalbC mice were treated with once or twice daily pazopanib for 4 weeks. Twenty four hours after the final dose, plasma samples were assayed for AR by ELISA. 5 mice/group. Bars, mean + SEM. 1-way ANOVA with Dunnett’s post test. 150 dose also analysed vs. vehicle by t-test with p value displayed. Doses utilized in preclinical anti-tumour experiments have included 25 mg/kg BID and 150 mg/kg SID, based on CEP analysis.
higher doses. It would be interesting to see if AR or other EGFR ligands are induced by treatment with a variety of VEGFR-2 inhibitors.

6.3 What is the optimal approach for combining chemotherapy with EGFR inhibitors?

There is not likely to be a simple single answer surrounding the best combinations for EGFR inhibitors and chemotherapy, and the optimal approach is likely to depend on the characteristics of the drugs involved, and/or type and stage of disease. Most chemotherapy drugs are cytotoxic and preferentially lethal to rapidly dividing cell populations. In contrast, EGFR inhibitors are considered cytostatic, particularly when utilized in a context where EGFR signalling is playing a direct role in tumour cell proliferation, as opposed to a pro-survival/anti-apoptotic role. The scheduling of these two types of agents, as reviewed by Kim & Tannock 101, should be taken into consideration, with the possibility of using a cytostatic EGFR agent between cycles of chemotherapy. The importance of scheduling the EGFR inhibitor gefitinib with the chemotherapeutic paclitaxel was recently investigated using the A431 carcinoma preclinical model 273. In vitro studies demonstrated superior results with concomitant treatment versus a sequential schedule that is highly sensitive to EGFR blockade. Simultaneous treatment approaches would be more easily accomplished with metronomic versus MTD chemotherapy regimens.

Given that our experiments with EGFR monoclonal antibodies and metronomic cyclophosphamide involved continuous exposure to both drugs, the
orthotopic primary tumour experiment was repeated with intermittent cyclophosphamide at the MTD – 100 mg/kg on days 1, 3, 5 of a 21-day cycle. Results show near equivalent effects when the chemotherapy schedules are compared (MTD vs metronomic). However, when combined with nimotuzumab, the MTD combination performed better, with plateaus of tumour growth corresponding to the days following the first week of each cycle (Figure 42). In this experiment the nimotuzumab was administered throughout the MTD regimen rather than waiting until the cyclophosphamide treatment was completed, yet the improvement in outcome with the targeted agent might involve suppression of repopulation within the tumour. Metronomic and MTD scheduling of chemotherapy need not be considered as “either/or” treatment choices, as approaches which merge these types of chemotherapy dosing could lead to sustained efficacy 274, although this type of approach would require dose reduction. Also, even if MTD scheduling of chemotherapy may result in superior efficacy when used in combination with EGFR targeted drugs, this treatment approach is not applicable for long term use, and lower dose, continuous metronomic chemotherapy regimens may be more appropriate in a maintenance setting. Their efficacy might be improved through pairing with an appropriate targeted drug, such as an antibody directed against EGFR. It is possible that targeted drugs may serve a unique purpose depending on how the chemotherapy drug with which they are partnered is given. The targeted drug may blunt an acute inflammatory-like, CEP, and/or tumour cell repopulation
Figure 42  Treatment of MDA-MB-231 variant 164/8-1B orthotopic xenografts with MTD or low-dose metronomic (LDM) cyclophosphamide in combination with nimotuzumab. Treatment began once xenografts reached 200mm³. MTD cyclophosphamide was administered at a dose of 100 mg/kg IP on day 1,3,5 of a 21-day cycle. LDM cyclophosphamide was administered at 20 mg/kg PO continuously in the drinking water. The dose of nimotuzumab was 500 mg every 3 days. 5 mice/group. Symbols and bars, mean + SEM.
following insults induced by MTD chemotherapy, and contribute to chronic cytostasis, including angiogenic suppression, during longer-term maintenance.
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Appendices

Appendix 1: The PC3 model for VEGFR-2 expression

<table>
<thead>
<tr>
<th>Cell line</th>
<th>VEGF (pg/ml/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC3</td>
<td>101.41</td>
</tr>
<tr>
<td>PC3.hcg.68</td>
<td>101.97</td>
</tr>
<tr>
<td>MDA-MB-435.hcg.17</td>
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</tr>
<tr>
<td>HT29.hcg.20</td>
<td>1222.74</td>
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</tbody>
</table>

**VEGF levels in conditioned media from various cell lines.** Human tumour cell lines were grown in monolayer culture. Human VEGF was assayed by ELISA and controlled for cell count.
Expression of VEGFR-2 protein on human PC3 prostate carcinoma cells. Western blot was performed on PC3 lysates by Dr. John Ebos.
Appendix 1

Human VEGF elevation in conditioned media upon treatment of PC3 with CT322 in vitro. Cells were incubated with the VEGFR-2 adnectin CT322 at the concentrations indicated for 24 hours. Conditioned media was analysed for human VEGF by ELISA, and plotted as % change from untreated cells. Experiment performed with Dr. John Ebos.
Appendix 2: Clinical phase I data with ramucirumab (IMC-1121b) reveals elevated circulating VEGF in serum of patients

Scatterplots depicting (A, C, and E) raw data and (B, D, and F) mean % changes from pretreatment values in the following pharmacodynamic assessments over time after first infusion of ramucirumab: (A and B) serum vascular endothelial growth factor (VEGF)-A; (C and D) serum soluble VEGF receptor (sVEGFR)-1; and (E and F) serum sVEGFR-2

Spratlin, J. L. et al. J Clin Oncol; 28:780-787 2010 – Figure 2.

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Appendix 3: Development and testing of preclinical models of colorectal cancer for evaluating metronomic chemotherapy combinations.

The subcutaneous GEO model treated with LDM cyclophosphamide and cetuximab. Single agent sensitivity of xenografts from this cell line is demonstrated with both cetuximab and nimotuzumab (top). There is minimal activity of cyclophosphamide chemotherapy alone, however combination treatment was more efficacious than cetuximab alone.
Development of an intrasplenic model with HT29 transfected with luciferase. Preliminary images of this model, taken with a xenogen camera. These cells also have βhcg for non-invasive monitoring of tumour burden. Cells prepared and experiments conducted with Dr. Giulio Francia.
Testing of the HT29 intrasplenic model with cyclophosphamide and cetuximab combination therapy. One million cells were inoculated into the spleen of SCID mice. Tumour burden was monitored by urinary hCG and luciferase imaging. Treatment began when hCG signal began to rise, at approximately 4 weeks. No improvement in tumour burden or survival was noted with therapy.
Testing of the HT29 intrasplenic model with cyclophosphamide, cetuximab and ME1 combination therapy. Drugs were combined to test the impact of host EGFR inhibition with ME1 in addition to tumour EGFR targeting by cetuximab. One million cells were inoculated into the spleen of SCID mice. Tumour burden was monitored by urinary hCG and luciferase imaging. Treatment began when hCG signal began to rise, at approximately 4 weeks. No improvement in tumour burden or survival was noted with therapy.
Second trial of intrasplenic HT29 with lower initial disease burden. Mild survival benefit was obtained with single agent cetuximab in this model. 6 mice in cetuximab group. 5 mice in control group.
Appendix 4: Clinical data with IGF-1R monoclonal antibodies reveals elevated circulating IGF-1 in serum of patients

Mean percentage change from baseline in total IGF-I and the R1507 serum concentration values following a single i.v.

Kurzrock R et al. Clin Cancer Res 2010;16:2458-2465 – Figure 2.
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Appendix 4

Mean (% of baseline +/- sd) serum IGF-1-time profiles of CP-751,871 in multiple myeloma patients after cycle 1 CP-751,871 treatment

Lacy, M. Q. et al. J Clin Oncol; 26:3196-3203 2008 – Figure 1 C.

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