# MECHANISMS OF REVERSIBLE SORAFENIB RESISTANCE IN HEPATOCELLULAR CARCINOMA

By

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# Abstract

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Conventional wisdom in oncology is that drug resistance is a stable and heritable phenotype due largely to cancer cell acquisition of genetic alterations. Thus, most cancer agents are given as sequential lines of therapy since continued use of the same agent is considered futile after disease progression. The anti-angiogenic tyrosine kinase inhibitor sorafenib is the only approved treatment for advanced hepatocellular carcinoma (HCC) patients. Its efficacy is further hindered by intrinsic (upfront) and acquired drug resistance (initial response and subsequent relapse) and a lack of alternative therapies. Our laboratory previously demonstrated using a human xenograft model of locally advanced HCC that acquired resistance to sorafenib is reversible due to re-sensitization of the tumour following transfer of resistant cancer cells to new hosts. An implication is that continued or rechallenge treatment with sorafenib could be effective strategies in HCC.

The mechanistic basis of reversible sorafenib resistance was investigated and was observed to be multifactorial. First, prompted by similar findings in HCC patients, tissue and plasma sorafenib levels of sorafenib were found to gradually decline over time in drug-resistant relative to sensitive tumour-bearing mice. These changes were partially attributed to tumoural induction of the sorafenib-metabolizing

enzyme CYP3A4. Escalating the dose to restore drug levels resulted in excessive toxicity and did not completely reverse drug resistance. Second, sorafenib treatment was found to effectively inhibit angiogenesis to the same extent in responding as well as resistant tumours. Rather, tumours hijacked or "co-opted" the host liver vasculature by becoming increasingly invasive and incorporating the adjacent parenchyma. Many of the changes associated with resistance were reversed by stopping treatment. Altogether, sorafenib treatment of HCC could be improved by 1) individualizing the dosage of drug; 2) prolonging anti-angiogenic treatment after disease progression; and 3) targeting co-opted vessels in conjunction with angiogenesis. From a survey of the literature, unstable forms of drug resistance may be applicable to other classes of anti-cancer therapy, including agents that directly target cancer cells. This phenomenon appears to be highly under-appreciated as a characteristic of cancers but offers important opportunities to extend the utility of many existing cancer drugs.

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# List of Abbreviations

| | |
|---|---|
| AIPC | Androgen independent prostate cancer |
| Ang | Angiopoietin |
| ATP | Adenosine tri-phosphate |
| BSA | Bovine serum albumin |
| BSC | Best supportive care |
| CYP3A4 | Cytochrome p450 3A4 |
| DAPI | Diamidinophenylindole |
| DMEM | Dulbecco's Modified Eagles Medium |
| DNA | Deoxynucleic acid |
| DMSO | Dimethyl sulfoxide |
| ECM | Extracellular matrix |
| ELISA | Enzyme-linked immuno-sorbent assay |
| EMT | Epithelial-to-mesenchymal transition |
| FBS | Fetal bovine serum |
| FGF | Fibroblast growth factor |
| G-CSF | Granulocyte-colony stimulating factor |
| GIST | Gastrointestinal stromal tumor |
| HBV | Hepatitis B virus |
| HCC | Hepatocellular carcinoma |
| hCG | Human chorionic gonadotropic hormone |
| HCV | Hepatitis C virus |
| HGF | Hepatocyte growth factor |
| HR | Hazard ratio |

| | |
|---|---|
| HRP | Horseradish peroxidase |
| IF | Immunofluorescence |
| IHC | Immunohistochemistry |
| (mi)RNA | Micro ribonucleic acid |
| MMP | Matrix metalloproteinase |
| NSCLC | Non-small cell lung carcinoma |
| OPN | Osteopontin |
| PBS | Phosphate-buffered saline |
| PDGF(R) | Platelet-derived growth factor (receptor) |
| PD-(L)1 | Programmed death (ligand) 1 |
| PE | Peak enhancement |
| PFS | Progression-free survival |
| PlGF | Placental growth factor |
| PR | Partial response |
| PSA | Prostate-specific antigen |
| RECIST | Response evaluation criteria in solid tumours |
| (q)RT-PCR | (Quantitative) reverse transcription-polymerase chain reaction |
| RCC | Renal cell carcinoma |
| SCID | Severe combined immunodeficient |
| SD | Stable disease |
| SDF | Stromal-derived factor |
| TKI | Tyrosine kinase inhibitor |
| UGT | UDP-glucuronosyltransferases |
| VEGF(R) | Vascular endothelial growth factor (receptor) |
| WiR | Wash-in rate |

# Chapter 1. Introduction

## MAJOR CARCINOGENESIS PATHWAYS

When normal cells become malignant, they gradually acquire characteristics that allow them to grow and ultimately spread throughout the body, unchecked by normal homeostatic mechanisms. The unique traits of cancer cells promote progression of the tumour but also offer potential targets to control the disease. At least eight 'hallmarks' of cancer have been described following decades of cancer research, which characterize the uniting features of malignancies[1] (Figure 1.1). There is no doubt that cancer is a genetic disorder originating from mutations in the DNA sequence of a cell, and historically, the focus of research attention and therapeutic intervention has been on the cancer cell itself. However the normal host tissues and tumour stroma have been increasingly identified as permissive to the growth and progression of a tumour. Thus, the host may not necessarily be a passive bystander, but become recruited or hijacked by the tumour to promote disease progression[2].

### Deregulated cell death, growth and proliferation

Four of the original six hallmarks of cancer[3]: resistance to cell death, sustained proliferation, evasion of growth-suppressive signals and indefinite replicative capability, are features that allow tumour cells to grow and survive beyond the capacity of normal cells. Deregulated growth factor signaling in particular is a central mechanism of tumour cell growth and proliferation. These are typically conveyed by surface growth factor receptors that communicate via intracellular tyrosine kinase domains and are frequently activated as a result of somatic mutations in upstream growth factors receptors (e.g. epidermal growth factor receptor, EGFR) or downstream cell signaling proteins (e.g. Raf or K-Ras)[1]. Such genetic aberrations lead cells to become unresponsive to growth factor signals and chronically in an activated, growth-promoting state. The signals which deregulate cell growth and proliferation may also be host-driven, rather than being intrinsic to the cancer cell. Tumour associated fibroblasts become activated by tumour cells and express abundant levels of pro-inflammatory and growth factors which may act in a

paracrine fashion on cancer cells to directly stimulate their growth[4]. Many of the approved molecular targeted therapies such as cetuximab and erlotinib (EGFR-targeted), trastuzumab (HER2), vemurafenib (Raf) serve to block growth factor-dependent tumour growth and signaling cascade proteins. Conventional chemotherapy (e.g. paclitaxel, cisplatin, 5-Fluorouracil) takes advantage of the rapid proliferation rate of cancer cells by directly damaging the DNA or regulators of DNA synthesis, thereby causing cytotoxicity.

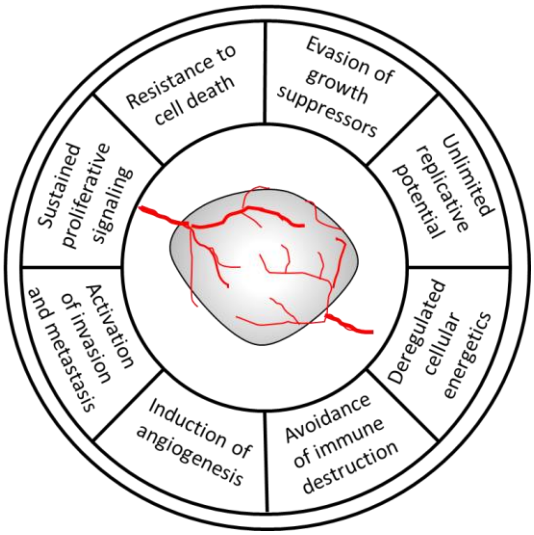


**Figure 1.1** The hallmarks of cancer. There are at least eight uniting features of all malignancies, and further tumour-promoting traits, such as inflammation and genome instability and mutation, that contribute to tumour progression. These also provide opportunities for therapeutic intervention. Figure redrawn from reference[1] by E.A. Kuczynski.

## Deregulated energy metabolism and evasion of the immune system

Altered cellular energy metabolism is recognized as one of the more recent cancer hallmarks. Cancer cells tend to favour glucose metabolism to pyruvate and subsequently to lactate in the cytoplasm, largely bypassing the oxygen-consuming oxidative phosphorylation step in the mitochondria[1]. This capability appears to be mediated largely by activated oncogenes and mutated tumour suppressors, which

promote tumour growth and survival[1,5]. Glycolytic metabolism is inefficient for energy production and cells compensate by increasing their rate of glucose consumption (e.g. by increasing expression of GLUT1 glucose transporters)[6]. This process is pronounced in hypoxic tumour microenvironments through hypoxia-inducible factor (HIF)1-α and HIF2-α transcription factor-mediated up-regulation of glycolytic enzymes[6].

A new hallmark, in which tumour cells evade elimination by the host immune system, is a promising new therapeutic target[1]. The immune system is designed to survey tissues and localize and destroy foreign or mutated self-antigens. Generally this suppresses tumour development, however tumours may dampen this response by recruiting immunosuppressive inflammatory cells (e.g. myeloid-derived suppressor cells or regulatory T cells), expressing immunosuppressive factors such as transforming growth factor (TGF)-β or interkeukin-10, or down-regulating expression of immunogenic tumour antigens including MHC class I and co-stimulatory molecules[5]. To this end, cancer immunotherapy using immune checkpoint inhibitors such as monoclonal antibodies against CTLA-4, PD-1 or PD-L1 which block the immunotolerant functions of effector immune cells (T-lymphocytes, dendritic cells) is a therapeutic strategy that can cause remarkable anti-tumour responses in some patients[7-10]. Many clinical trials are underway which combine immunotherapy with other therapeutic modalities.

## Angiogenesis, invasion and metastasis

Another uniting feature of cancer is the ability of cancer cells to invade host tissues and metastasize to distant sites within the body. Metastasis occurs in a multi-step cascade beginning with local invasion, followed by detachment from other cells and the extracellular matrix (ECM), intravasation of cells into blood and lymphatic vessels, extravasation of cells into the parenchyma of a distant organ and development of new nodules. Cancer cells may also avoid the extravasation stem by adhering to endothelium, forming intravascular emboli and growing into new nodules[11]. Epithelial-to-mesenchymal

transition (EMT) is a normal reversible cellular development program during embryonic morphogenesis, but is commonly implicated (usually in the form of a partial EMT) in tumourigenesis and in the early invasion/metastasis steps. During EMT epithelial or carcinoma cells lose their apical-basal polarity, alter their cytoskeleton, change their cell shape to that of fibroblastic morphology and become more motile[12]. A reversal of this process (mesenchymal-to-epithelial transition, MET) may help disseminated cancer cells revert to an epithelial/carcinoma phenotype and help them become established in the new tissues. The role of EMT in tumour progression is however controversial and considered by some to be an artifact of the laboratory[13]. Host cells can also facilitate invasion and metastasis. For instance host stromal cells at the tumour interface may recruit macrophages, which may in turn facilitate invasion or intravasation into the vasculature by expressing matrix metalloproteinases (MMPs)[14].

The final hallmark feature of cancer is the ability to induce angiogenesis, which is defined as the formation of new blood vessels from a pre-existing vascular bed[1,15]. Angiogenesis is applicable to local primary tumours as well as new and established metastases. Tumour angiogenesis is described in detail below. As discussed in Chapter 3, while the ability of a tumour to acquire a vascular supply is mandatory in cancer, angiogenesis *per se* is not.

## TUMOUR ANGIOGENESIS

All tumours rely on a functional vascular supply for the delivery of nutrients and oxygen, and the removal of waste products. This theory was put forward by Dr. Judah Folkman in 1971 when he postulated that tumours require an expanding vascular supply if they are to grow beyond 1-2$mm^3$ in diameter, metastasize and ultimately become lethal[16]. Without angiogenic induction, tumours were said to be avascular and remain dormant in size. Through a local imbalance of pro- and anti-angiogenic factors,

the ‘angiogenic switch’ must be flipped in favour of the former thus causing induction of vessel growth and resultant rapid tumour growth sustained by perfusion[17].

The microcirculation develops through either vasculogenesis or angiogenesis. Vasculogenesis is the *de novo* formation of vessels, which occurs during embryogenesis through the differentiation and coalescence of endothelial precursors (angioblasts) into a primary vascular plexus[15]. Adult vasculogenesis may also occur through the mobilization and incorporation of bone marrow-derived endothelial progenitor cells or hematopoietic precursor cells into an existing vascular plexus[18,19]. Angiogenesis is a remodeling of the vascular plexus and occurs during development and a few restricted physiological sites and processes during adulthood, such as in the corpus luteum and in the uterus during pregnancy. In pathology, angiogenesis is involved in wound healing, retinopathy, and in cancer.

There are two types of angiogenesis: intussusceptive growth (the splitting of one vessel into two), or endothelial sprouting (Figure 1.2), which is considered the most important for tumour growth[20]. Sprouting angiogenesis occurs in a series of coordinated steps[21]. The capillary endothelium first becomes activated by an angiogenic stimulus, becomes hyperpermeable and dilates. Perivascular cells become detached and the local basement membrane is partially degraded. Next, endothelial cells lose their intercellular attachments and begin to migrate toward the angiogenic stimulus. A leading tip cell is followed by proliferating stalk cells to form a sprout. New basement membrane is deposited and the endothelial cells become polarized and form a lumen within the sprout. Proliferating pericytes migrate and eventually surround and stabilize the new vessel. In tumours, angiogenesis is poorly regulated and therefore the resultant vasculature displays striking abnormalities compared to normal blood vessels. Tumour vessels may be leaky, highly tortuous and disorganized, have uneven vessel diameters and abnormal blood flow, blind ends, poor pericyte coverage, discontinuous basement membrane and are often haemorrhagic [22]. The neo-circulation is frequently unable to meet the metabolic demands of a tumour, which leads to regions of ischemia, hypoxia and necrosis.

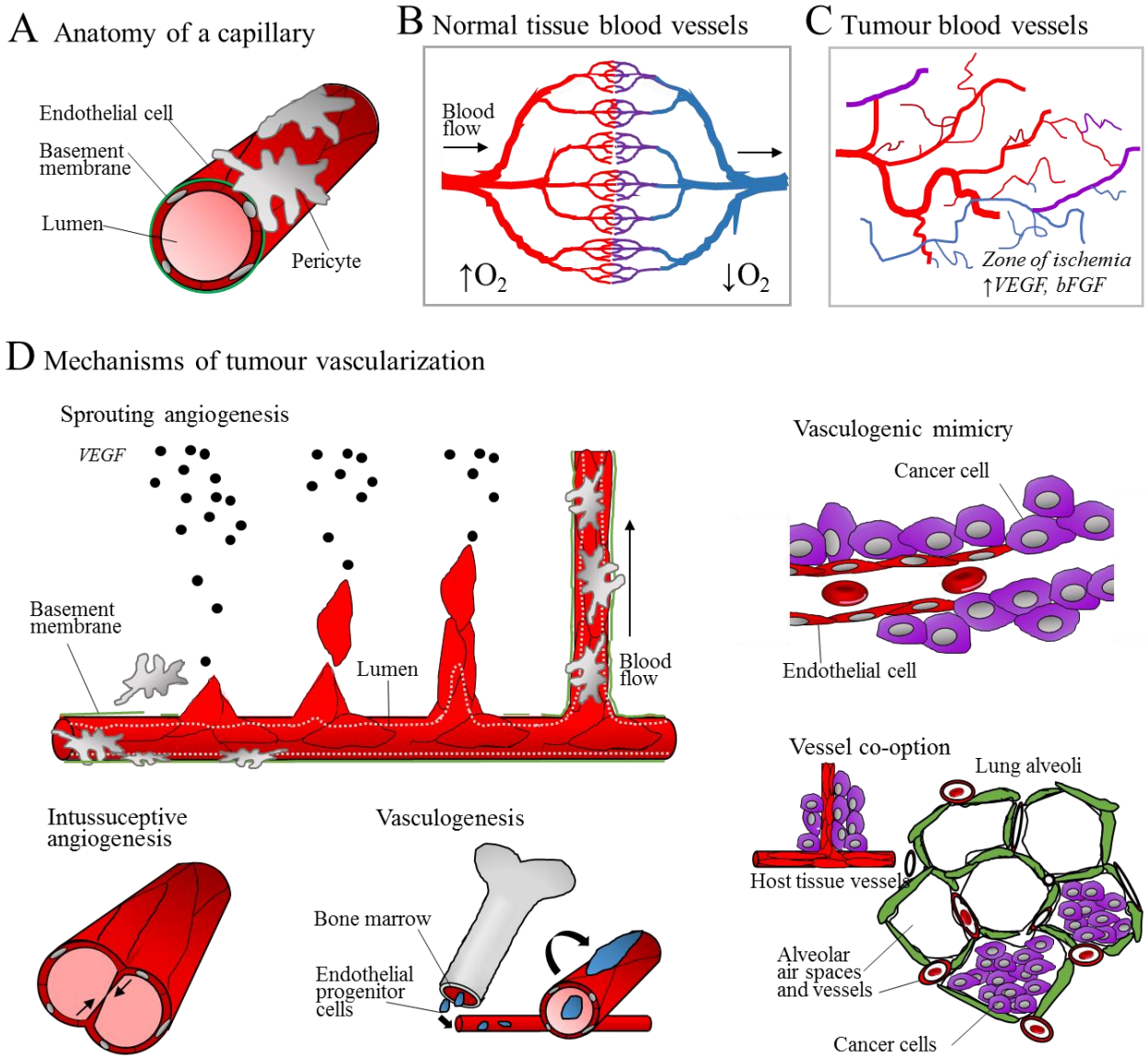


**Figure 1.1** The tumour vasculature. **A**. Structure of a normal vascular capillary. In contrast to physiological vessels, angiogenic blood vessels are poorly covered by extramural cells such as pericytes, have a discontinuous basement membrane and generally have poor structural and functional integrity. **B**. The normal vasculature of tissues forms a vessel hierarchy with unidirectional blood flow and well-perfused tissues. **C**. Tumour vessels are tortuous, disorganized and lead to tumour regions of ischemia and necrosis. **D**. The primary mechanism of tumour vascularization is sprouting angiogenesis. New vessels may also form via intussuceptive angiogenesis or the homing of endothelial progenitor cells from the bone marrow to sites of vessel formation. Alternative modes of tumour vascularization are vasculogenic mimicry and vessel co-option. Illustrations by E.A. Kuczynski.

All or most cancers are angiogenesis-dependent for growth. However as the variability in response to anti-angiogenic therapy suggests[23], the extent of angiogenic dependency varies considerably across malignancies. Alternative modes of tumour vascularization have been proposed. Cancer cells have been found to undergo 'vasculogenic mimicry' by forming vascular channels containing a basement membrane but unlined by endothelial cells[24]. The existence of vasculogenic mimicry is controversial and has been debated to be an artefact rather than a true vascular structure[25]. Vasculogenic mimicry may be limited to only a few aggressive tumour types such as melanoma[24]. Tumour vessel 'co-option' is a second alternative to tumour angiogenesis in which tumour cells migrate along and hijack the pre-existing normal host vasculature rather than induce new vessel growth[26]. The evidence for vessel co-option in human clinical specimens is mounting, and has been identified in liver metastases of colorectal and breast carcinomas[27], non-small cell lung carcinomas and lung metastases[28,29] and hepatocellular carcinoma[30] among others[26]. Therapeutic approaches to block the tumour vasculature are presently focused on targeting angiogenesis.

## Vascular endothelial growth factor signalling

The theory that angiogenesis is critical for tumour growth led to the discovery of endogenous stimulators and inhibitors of angiogenesis. Among the regulators of angiogenesis including bFGF, osteopontin (OPN), platelet-derived growth factor (PDGF), deltalike ligand (Dll) 4, angiopoietin-1 and -2, vascular endothelial growth factor (VEGF) is considered the most important for physiological and pathological angiogenesis[31]. The VEGF family is made up of five mammalian glycoproteins: VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF)-1. VEGF binds to three receptors in distinct but overlapping fashions: VEGF-A, -B and PlGF bind VEGFR-1; VEGF-A, -C, -D and VEGF-A/PlGF heterodimers bind VEGFR-2; and VEGF-C and -D bind VEGFR-3[32]. At least four VEGF-A splice variants exist in humans consisting of 121, 165, 189 or 206 amino acids, although $VEGF_{165}$ is the most commonly expressed[32,33]. VEGFR-2 is primarily expressed on endothelial cells and is the most

important positive mediator of angiogenesis, particularly in response to VEGF-A stimulation[34]. Homozygous deletion of VEGFR-2 is embryonic lethal in mice at E8.5-9.5 due to defects in blood island formation and hematopoeisis, therefore suggesting that VEGFR2 is critical for early vasculogenesis[35]. VEGFR-1 has a stronger affinity for VEGF than VEGFR-2, but weak kinase activity suggesting that it serves as a decoy receptor[36], but it also mediates monocyte migration, mobilization of endothelial progenitor cells and hematopoietic cell survival[37]. VEGFR-3 is expressed by lymphatic endothelium and is important for lymphangiogenesis[38].

The dominant theory of tumour angiogenesis is that cancer cells express VEGF which directly stimulates endothelial cell proliferation, survival and migration. Growth factors, cytokines and mutations in oncogenes or tumour suppressors may stimulate VEGF expression in tissues[31,39]. Hypoxia mediated by the transcription factors HIF-1α or HIF-2α, is one of the most important regulators of VEGF expression and serves as a mechanism to improve tissue perfusion and oxygenation[40]. VEGF-A elicits its effects by binding to the extracellular domain of VEGFR-2 which causes receptor dimerization and auto-phosphorylation at specific tyrosine residues on the intracellular domain[41]. Phosphorylation of Tyr1175 simulates endothelial cell proliferation via the Raf-MEK-ERK pathway[42] and endothelial cell migration by activation of phosphatidylinositol 3'-kinase (PI3K)[43]. Activated PI3K promotes cell survival by activating Akt, which inhibits pro-apoptotic proteins BAD and Caspase-9[44]. Phosphorylation of Tyr1214 activates Cdc42-p38 MAPK-dependent migration[45]. Finally, carboxy-terminal autophosphorylation activates focal adhesion kinase (FAK), which stimulates endothelial cell migration[46].

## ANTI-ANGIOGENIC THERAPY

Over the last two decades a large number of anti-angiogenic therapies have been developed for the treatment of cancer. These agents predominantly target the VEGF/VEGFR2 signaling cascade, although next-generation anti-angiogenic agents under development target alternate pro-angiogenic pathways including bFGF and angiopoietin signaling[47,48]. The first approved anti-angiogenic agent was bevacizumab (Avastin) in 2004 for the treatment of metastatic colorectal carcinoma. Since then nearly a dozen anti-angiogenic agents have been approved by the FDA for cancer treatment (Table 1). These agents include a) monoclonal antibodies against VEGF (e.g. bevacizumab) or VEGFR2 (ramucirumab); b) protein "Trap" molecules (decoy receptors; e.g. aflibercept), or tyrosine kinase inhibitors (TKIs, e.g. sorafenib, sunitinib and regorafenib).

### Mechanism of action of anti-angiogenic agents

While monoclonal antibodies and Trap protein molecules are target-specific, leading to few off-target effects and potentially less toxicity, TKIs are selective inhibitors of tyrosine and other kinases. Using sunitinib as an example, TKIs inhibit other VEGF receptors (e.g. 1 and 3), PDGF receptors, and other growth factor receptors (e.g. stem cell factor receptor (c-Kit) and colony stimulating factor (CSF) receptor)[49]. TKIs may potentially increase anti-tumour efficacy by acting on multiple cancer cell-expressed targets and angiogenic pathways simultaneously, but concept is often debated[50,51]. Bevacizumab is approved only in combination with chemotherapy (except for ovarian carcinoma). A controversial theory to explain why anti-VEGF therapy synergizes with chemotherapy is that VEGF inhibition leads to temporary improvements in vessel function or 'normalization' of the vasculature[52]. This is proposed to improve the delivery of administered chemotherapy and hence the anti-tumour effect. However recent studies in patients show that anti-angiogenic treatment *impairs* rather than improves the delivery of concurrent therapy[53,54]. In contrast to monoclonal antibody or Trap molecule treatments, TKIs

generally have shown success as monotherapy treatment[23]. Drug combinations with TKIs are generally too toxic, but another explanation may relate to the stromal cell content and its association with blood vessels in TKI vs. antibody-sensitive tumour types[55].

## Toxicities of anti-angiogenic treatment

Unlike conventional chemotherapy, which is typically administered according to a maximum tolerated dose, anti-angiogenic agents are administered at relatively low doses continuously until disease progression. Most of the adverse effects of anti-angiogenic therapy are mechanism-based and due to blockade of the VEGF pathway. They include hypertension, disrupted coagulation, impaired wound healing, gastro-intestinal perforations, hemorrhage, proteinuria, and thrombosis[56]. TKIs, which are oral drugs, are also associated with skin reactions such as hand-foot skin-reaction (HFSR) and diarrhea. Most often adverse events are minor and can be managed by brief therapy interruptions or dose reductions. Only in rare cases do they necessitate permanent treatment cessation. Accumulating evidence has found that the toxicities brought on by anti-VEGF treatment (in particularly oral TKIs) are related to drug exposure (plasma) levels[57,58]. Further studies have linked anti-VEGF drug exposure levels to therapeutic outcome[58]. As explored in Chapter 2, there is a viewpoint that anti-angiogenic therapy should not be given using a 'one-size-fits' all approach, but rather should be optimized and tailored to each individual patient to account for differences in drug absorption, distribution, metabolism and excretion.

**Table 1.1** Anti-angiogenic therapies that are approved for the treatment of cancer.

| Agent | Type | Mechanism of action | Clinical Indications | First year of approval | Company |
|---|---|---|---|---|---|
| Bevacizumab (Avastin) | Humanized monoclonal antibody | Antibody against VEGF-A | Metastatic CRC, NSCLC, RCC, recurrent GBM, recurrent epithelial ovarian cancer, cervical, fallopian tube or primary peritoneal cancer | 2004 | Genentech/ Roche |
| Sorafenib (Nexavar) | Small molecule TKI | Inhibitor of VEGFRs, Raf, PDGFRs, KIT | Metastatic RCC, hepatocellular carcinoma, radioactive iodine-refractory thyroid cancer | 2005 | Bayer/Onyx |
| Sunitinib (Sutent) | Small molecule TKI | Inhibitor of VEGFRs, PDGFRs, FLT-3, CSF1R | Metastatic RCC, imatinib-resistant GIST, pancreatic neuro-endocrine tumours | 2006 | Pfizer |
| Pazopanib (Votrient) | Small molecule TKI | Inhibitor of VEGFRs, PDGFRs, KIT | Metastatic RCC, soft tissue sarcoma | 2009 | GlaxoSmith Kline |
| Vandetanib (Caprelsa) | Small molecule TKI | VEGFRs, PDGFRs, EGFR | Metastatic medullary thyroid cancer | 2011 | AstraZeneca |
| Axitinib (Inlyta) | Small molecule TKI | Inhibitor of VEGFRs, PDGFRs, KIT | Metastatic RCC | 2012 | Pfizer |
| Ziv-Aflibercept (Zaltrap) | Chimeric soluble receptor | Binds VEGF-A, VEGF-B, PlGF | Metastatic CRC | 2012 | Regeneron/S anofi Aventis |
| Cabozantinib (Cometriq) | Small molecule TKI | Inhibitor of VEGFRs, PDGFR, cMET, RET, KIT | Progressive medullary thyroid cancer | 2012 | Exelixis |
| Regorafenib (Stivarga) | Small molecule TKI | Inhibitor of VEGFRs, Raf, PDGFRs, Tie2 | Metastatic CRC, Locally advanced or metastatic GIST | 2012 | Bayer/Onyx |
| Ramucirumab (Cyramza) | Humanized monoclonal antibody | Antibody against VEGFR2 | Metastatic NSCLC, advanced gastric adenocarcinoma | 2014 | ImClone/Eli Lilly |
| Lenvatinib (Lenvima) | Small molecule TKI | Inhibitor of VEGFRs | Radioactive iodine-refractory thyroid cancer | 2015 | Eisai |

Abbreviations: TKI = tyrosine kinase inhibitor; CRC = colorectal carcinoma; RCC = renal cell carcinoma; GBM = glioblastoma multiforme; GIST= gastrointestinal stromal tumour; NSCLC = non-small cell lung carcinoma.

References:[59], http://www.fda.gov/Drugs/InformationOnDrugs/ApprovedDrugs/

On one hand, Judah Folkman's theory that all lethal tumours depend on angiogenesis for growth has been validated by the incorporation of anti-VEGF agents into standard of care therapies for a range of tumour types. Current anti-angiogenic agent approvals are for colorectal carcinoma, non-small cell lung carcinoma, hepatocellular carcinoma, renal cell carcinoma, ovarian carcinoma, cervical cancer, glioblastoma multiforme, gastrointestinal stromal tumours and pancreatic neuroendocrine tumours (www.cancer.gov). On the other hand, the degree of clinical benefit is typically modest or incremental[23,60]. In responding patients, tumours rarely regress. Rather, anti-angiogenic agents typically stabilize tumour growth (documented as stable disease, SD) and less frequently lead to regression (typically a partial response, PR). In many cases they slow progression as indicated by increased progression-free survival (PFS) times.

## RESISTANCE TO ANTI-ANGIOGENIC THERAPY

Therapeutic drug resistance, or loss of responsiveness to a given anti-cancer therapy, is a leading cause of treatment failure. Originally it was postulated that since anti-angiogenic agents target host cells which are devoid of genetic instability (unlike cancer cells), resistance to therapy would not develop in response to these agents[61]. However, tumour endothelium in mouse models and human carcinomas has been found to contain chromosomal abnormalities[62,63]. Moreover, experience has shown that anti-angiogenic therapy is not exempt from drug resistance. There are two modes of resistance to anti-angiogenic therapy that have been described. The first, acquired resistance (also known as evasive or adaptive resistance), occurs when a tumour initially responds to therapy but eventually adapts to therapy and progresses. The second form, termed intrinsic resistance (also known as primary or upfront resistance), occurs when a tumour does not respond to therapy[64] (Figure 1.3).

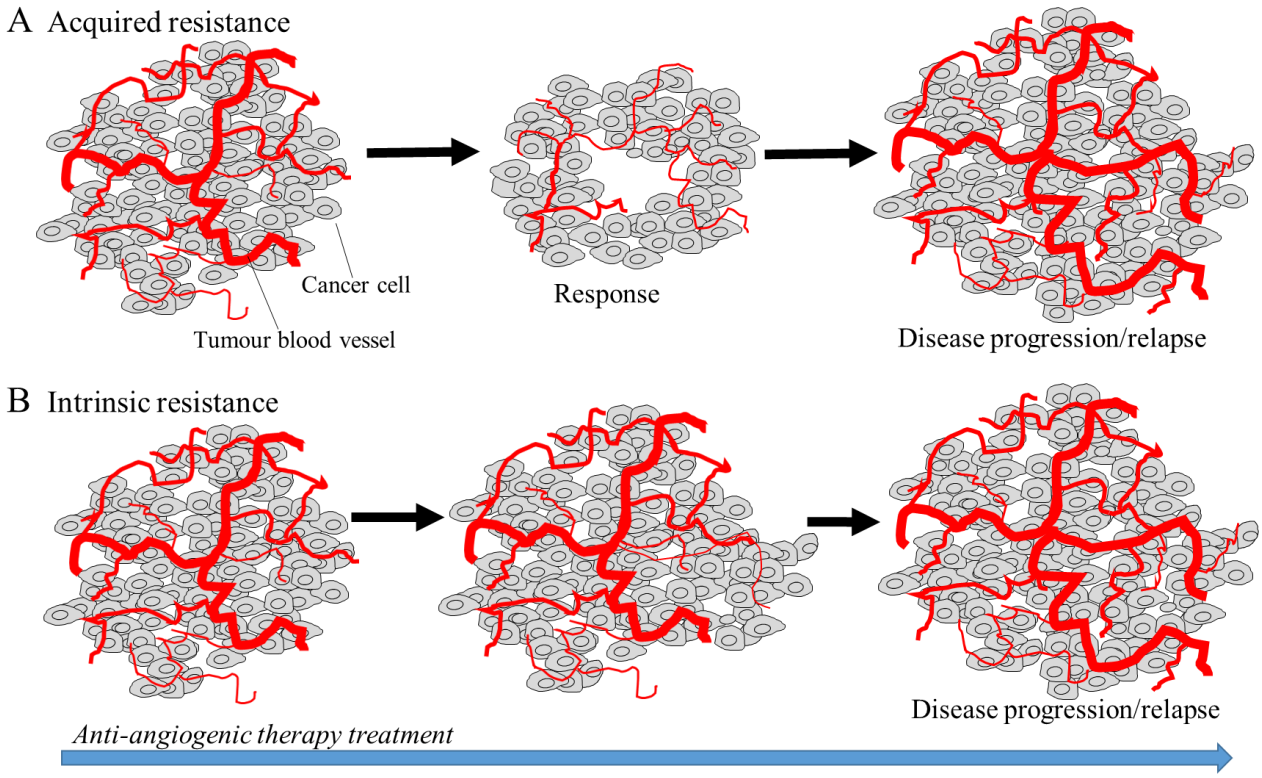


**Figure 1.2** Major forms of resistance to anti-angiogenic therapy. **A**. During acquired resistance tumours initially respond to anti-angiogenic therapy, which is typically characterized by reduced tumour vascularity, necrosis within the tumour core and stabilized tumour growth. Following an initial therapeutic response tumours relapse on therapy, which is often attributed to a rebound of the vasculature. **B**. During intrinsic resistance tumours have an innate lack of sensitivity to therapy and progress regardless of treatment. Figure redrawn from reference[64] by E.A. Kuczynski.

## Tumour vessel heterogeneity

The mechanisms to explain resistance to anti-angiogenic therapy are numerous (Figure 1.3). Tumour vessel heterogeneity may contribute significantly to the impact of anti-angiogenic treatment. For instance, the existing tumour vasculature may adapt to VEGF inhibition by down-regulation of VEGFR2 expression on endothelial cells[65]. During anti-angiogenic treatment tumour endothelium may recruit more pericytes to become more stabilized and thus insensitive to therapy[66]. Pericytes have been shown to

survive anti-VEGF treatment leaving behind empty basement membrane sleeves, which endothelial cells repopulate upon cessation of treatment[67]. Recently, at least five different vessel subtypes have been found in human tumours including mother vessels, glomeruloid microvascular proliferations, vascular malformations, feeding arteries and draining veins[68]. Mother vessels and glomeruloid microvascular proliferations, which are vessels that develop early during tumour progression, were found to be most sensitive to anti-VEGF treatment while the other subtypes were mostly resistant. This suggests that many established tumour vessels may be unresponsive to current anti-angiogenic (anti-VEGF) treatment strategies[68].

## Re-induction of angiogenesis

The majority of mechanisms to explain drug resistance involve a strategy by which the tumour 'escapes' angiogenesis inhibition by re-establishing a vascular supply[60]. One of the most important and accepted mechanisms of resistance (particularly for acquired resistance) involves re-activation of angiogenesis through induction of hypoxia-responsive compensatory circulating pro-angiogenic factors[60]. Up-regulation of compensatory factors ephrins, members of the fibroblast growth factor family (FGF)[69,70], angiopoietin-1 (Ang1)[69], interleukin-8[71], HGF[72], PDGF-C[73] and VEGF-C[74] have each been implicated in driving resistance to anti-VEGF therapy. Down-regulation of angiostatic chemokines such as interferon-γ and CXCL9 to 11 in resistant tumours[75] or recruitment of pro-angiogenic myeloid cells resulting from up-regulated pro-inflammatory cytokines (such as SDF-1, G-CSF and GM-CSF) have also been implicated in the escape process[60,76-78]. These circulating factors are not blocked by first generation VEGF inhibitors but targeting both VEGF plus a putative resistance factor, be it Ang1[79], Ang2[80], bFGF[69,81], PlGF[82] or HGF[72], has shown great promise in preclinical models by way of enhancing anti-tumour activity compared to targeting VEGF alone. Anti-angiogenic treatment can also lead to recruitment of bone marrow-derived endothelial progenitor cells into the tumour vasculature, which can contribute to tumour revascularization[83].

## Increased cancer cell invasiveness and metastasis

A troubling side-effect of anti-angiogenic treatment, at least in preclinical tumour models, is the ability to increase the aggressiveness of cancer cells. This may be an escape mechanism of hypoxic cancer cells to occupy more oxygenated and nutrient-rich niches at local or distant organ sites[84]. Although sustained treatment with sunitinib inhibited tumour growth in established mouse syngeneic and xenografted tumours, short-term treatment prior to (neoadjuvant-like therapy) and immediately following (adjuvant therapy) intravenous administration of cancer cells resulted in an increased rate of multi-organ site metastasis and decreased overall survival compared to controls[85]. Treatment of RIP-Tag genetically engineered pancreatic neuroendocrine tumour-bearing mice and glioblastoma xenografted mice with anti-VEGFR2 monoclonal antibody DC101 (or sunitinib) showed that following a brief anti-tumour and anti-angiogenic effect lasting one week, there was increased tumour invasion during and following treatment in addition to increased metastasis[86]. Studies have shown that treatment-induced EMT (including expression of molecular markers for EMT and morphological changes of cells) could be a mechanism that contributes to increased invasiveness or metastasis[87-89]. However the observed promotion of metastasis *prior* to tumour induction[85] also implicates a host-dependent mechanism, such as induction of circulating proangiogenic cytokines and growth factors which could promote the formation of a pre-metastatic niche[90,91].

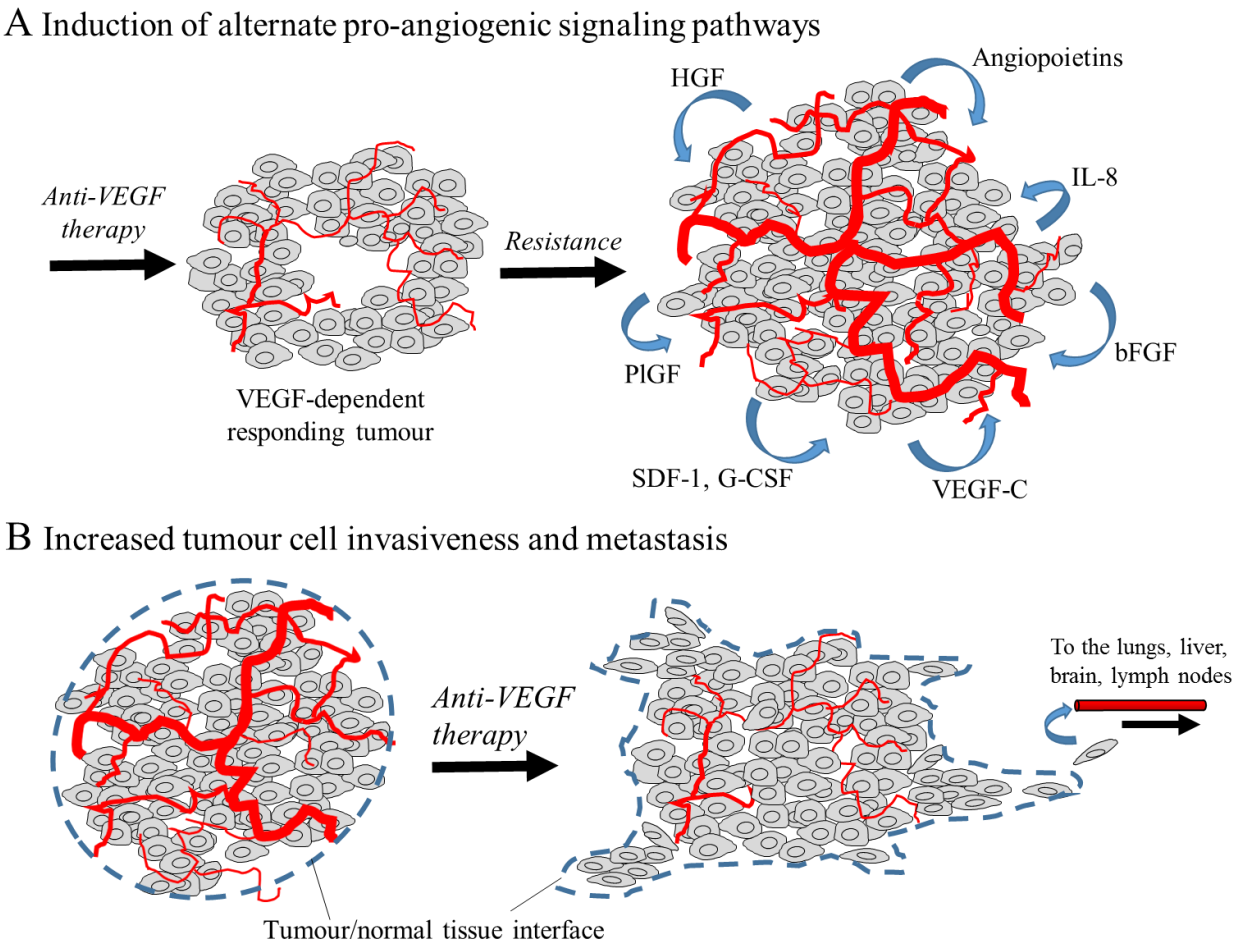


**Figure 1.3** Major proposed resistance mechanisms to anti-angiogenic therapy. **A**. Anti-angiogenic (anti-VEGF) treatment has been shown to lead to induction of alternate compensatory pro-angiogenic growth factors and cytokines in the tumour and/or host, including those that stimulate angiogenesis directly (e.g. PlGF or bFGF) or indirectly (e.g. SDF-1 or G-CSF via recruitment of bone marrow-derived cells). VEGF-independent rebound revascularization therefore leads to tumour regrowth and disease progression. **B**. Anti-angiogenic therapy has also shown to induce a more aggressive and invasive phenotype in cancer cells, which promotes infiltrative growth into the surrounding tissues (dashed line) and a greater potential for metastasis. Tumour-independent pro-metastatic effects have also been proposed. Figure redrawn from reference[64] by E.A. Kuczynski.

The clinical relevance of the above resistance mechanisms is not clear. Circulating biomarkers, among them levels of VEGF, PlGF and bFGF, have failed to be associated consistently with treatment outcome[23]. Phase III clinical trials have failed to demonstrate the superiority of targeting resistance mechanisms[92-95] or potentiating inhibition of VEGFR using more selective inhibitors[96,97] compared to VEGF inhibition alone. In contrast to preclinical studies, pre-existing advanced metastatic disease usually does not become more aggressive in patients treated with angiogenesis inhibitors[98,99]. The duration of treatment as well as stage of disease (early microscopic vs. late metastatic) may influence the pro-invasive or pro-metastatic effects of anti-angiogenic agents, but the clinical relevance of this phenomenon is still up for debate[100].

## ANTI-ANGIOGENIC TREATMENT OF HEPATOCELLULAR CARCINOMA

### Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related deaths worldwide. HCC was responsible for approximately 750,000 deaths in 2012, most of which were in developing countries[101]. The 5-year survival rate for HCC patients is among the lowest at approximately 10%[102]. Liver cancer encompasses several histologically distinct cancers, the most common of which are HCC, childhood hepatoblastoma, adult cholangiocarcinoma (cancer of the intrahepatic bile ducts) and angiosarcoma (cancer of the intrahepatic blood vessels)[103]. HCC is by far the most common of liver cancers representing approximately 90% of all cases[102]. HCC rates are approximately twice as high in men as in women[104]. HCC occurs at a relatively low rate in North America compared to many other countries such as China, however over the last few decades age-adjusted incidence rates have increased in

low-incidence countries such as Canada. In Western countries HCC mortality rates have also increased faster than for any other leading type of cancer[104]. Thus, HCC is a significant and growing problem.

Chronic infections with hepatitis B or C virus are the primary risk factors for HCC[103,105]. HBV infection is endemic in China, Southeast Asia and sub-Saharan Africa whereas hepatitis C virus is more frequent in North America. Obesity, type II diabetes, alcohol consumption, smoking and aflatoxin hepatotoxin are other risk factors that may lead to chronic liver disease and HCC. Typically HCC develops on a background of liver cirrhosis, which is a fibrotic reaction in the liver characterized by excessive ECM production and is associated with impaired liver function[106]. Cirrhosis can occur from acute damage by a toxin or hepatitis virus, leading to chronic inflammation, necrosis and liver regeneration. From cirrhotic tissue, cellular dysplasia and HCC may develop through the acquisition of genetic alterations[107].

## Mechanisms of hepatocarcinogenesis

Multiple molecular pathways have been identified as important for hepatocarcinogenesis. In viral induced HCCs, HBV can directly promote transformation by integrating into the genome by causing genetic microdeletions in cancer-causing genes or by viral gene (e.g. HBx) integration and alteration of key cell cycle and growth genes such as SRC tyrosine kinases, Ras, Raf and ERK[106]. Host factors including altered immune responses (overactive, damaging cytotoxic T cell responses during early development and immune evasion of HBV, HCV or tumour cell clearance at later stages) and activation of hepatic stellate cells (which contribute to deposition of ECM and fibrosis) also contribute to HCC development[108,109]. Genetic events that contribute to HCC development include chromosomal alterations, inactivation of p53, overexpression of HGF receptor, overexpression of ErbB receptor family members (including EGFR) and mutations in β-catenin[106].

Angiogenesis is considered particularly important for HCC progression and indeed HCCs are hypervascular tumours[110]. During HCC development, liver sinusoids undergo 'capillarization' where they are remodeled into neovessels and express CD34, a molecular marker of immature angiogenic vessels[111]. Through this process the vasculature of HCCs switches from a blood supply provided primarily from the portal vein (and the gut) in the normal liver to supply by the hepatic artery. These changes in liver perfusion aid in the detection of HCC nodules using contrast-enhanced imaging techniques.

VEGF appears to be a driver of angiogenesis during early and late steps of HCC development. Upregulated VEGF expression has been observed in cirrhotic relative to non-cirrhotic livers[112], and progressively increasing VEGF levels have been found from low-grade dysplasia to high-grade dysplasia to early-stage HCC[113]. Increased plasma concentrations of VEGF as well as other pro-angiogenic growth factors such as angiopoietin-2 and PDGF-B have been observed in HCC patients relative to cirrhotic patients[114]. High VEGF is also associated with high tumour grade, vascular invasion, portal invasion and rapid tumour recurrence[110]. VEGF signaling is therefore considered a key driver of HCC tumour progression.

## Treatment of early and advanced stage hepatocellular carcinoma

There has been a frustrating lack of progress in HCC treatment. HCCs are intrinsically resistant to systemic chemotherapy[110], which sets it apart from many other cancers. Until recently, treatment options were restricted to HCC patients whose disease was diagnosed at an early stage. These treatments include liver resection, liver transplantation or local ablation techniques such as ethanol injection, radio-ablation or chemo-ablation[104] (Figure 1.5). Unfortunately, approximately 80% of patients present with advanced or unresectable disease at the time of diagnosis[115] meaning that most patients are ineligible for these treatments.

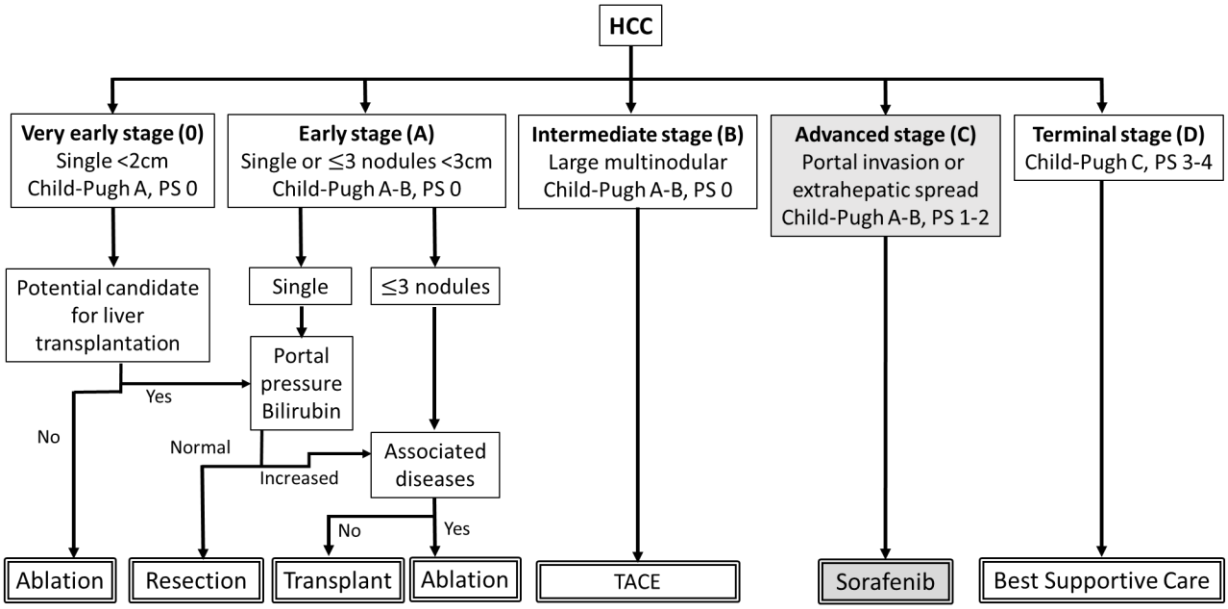


**Figure 1.4** Treatment algorithm for hepatocellular carcinoma. Treatments for early stage HCC are potentially curable, whereas treatment of intermediate to terminal stage is palliative. TACE = transarterial chemoembolization. Figure redrawn from reference[116].

The oral anti-angiogenic TKI sorafenib is the only systemic treatment shown to demonstrate a survival benefit in advanced HCC patients when given at standard doses of 400mg twice daily[117,118]. In the phase III SHARP trial in advanced HCC patients, sorafenib treatment extended median overall survival from 7.9 to 10.7 months and improved the median time to radiologic progression from 2.8 to 5.5 months compared to placebo treatment[117]. A second phase III trial in Asian patients (the Asia-Pacific trial) also observed a significant improvement in median overall survival with sorafenib treatment from 4.2 to 6.5 months[118]. The differences in outcome between the two trials are likely a consequence of regional difference in etiological factors (73% and 12.0% of patients were infected with HBV at baseline in the Asia-Pacific and SHARP trials, respectively, and 8.4% and 30% were infected with HCV, respectively)[118]. On the basis of these trials sorafenib was approved by the FDA in 2007 for treatment of advanced HCC.

Sorafenib is a multikinase inhibitor of Raf serine/threonine kinases and the receptor tyrosine kinases VEGFR2 and 3, PDGFRβ, Flt-3 and c-Kit[119]. Thus, sorafenib is proposed to possess anti-angiogenic activity against stromal endothelial and pericyte cells (by VEGFR and PDGFR inhibition, respectively) and targets cancer cells directly by inhibiting among others the Raf/MAPK pathway, which is an upstream stimulator of cellular proliferation[120]. Inhibition of Raf signaling may also contribute to the anti-angiogenic effect in endothelial cells. Recently, Raf-independent pro-apoptotic activity of sorafenib has been observed in cancer cells. This appears to be mediated by inhibition of STAT3 signaling[121,122].

## Limitations of current HCC treatments

Based on phase III trial data, an estimated 35-43% of HCC patients' tumours are sensitive to sorafenib treatment[117,118]. Toxicities are common in sorafenib-treated patients – particularly hand-foot skin reaction (HFSR), diarrhea and fatigue. Rarely do they lead to permanent therapy discontinuation[117,118]. Therefore most patients that remain on sorafenib therapy will ultimately display intrinsic or acquired resistance. Once this occurs the convention in oncology is to permanently stop therapy once disease progression has been noted. Disease progression is defined using a set of criteria such as RECIST (Response Evaluation Criteria in Solid Tumours) or more recently modified (m)RECIST, which uses whole tumour volume changes or viable tumour volume changes, respectively, but the optimal assessment criteria are still under investigation[123]. There are no proven therapies as alternatives to sorafenib or as second line therapy. Seven phase III clinical trials have been conducted in the first-line or second-line setting in patients with advanced HCC since the approval of sorafenib and each trial was negative (Table 1.2). Five of these trials have evaluated alternative anti-angiogenic agents including TKIs (sunitinib, brivanib, linifanib) and a monoclonal antibody against VEGFR2 (ramucirumab). Thus sorafenib remains the preferred drug[95]. The efficacy of sorafenib appears to be restricted to late, metastatic disease. The recently completed phase III 'STORM' trial evaluated sorafenib

treatment in early stage micrometastatic disease post-tumour resection (adjuvant setting), but no improvement of recurrence-free survival was achieved by sorafenib compared to placebo treatment[124].

The repeated failure of trials in HCC is paralleled by the failure of a number of randomized phase III adjuvant trials involving antiangiogenic drugs. The drugs include bevacizumab with chemotherapy in breast and colorectal cancer and sorafenib or sunitinib in renal cell carcinoma (RCC). In each case the clinical trials failed to demonstrate a benefit despite prior successes of the same drugs in the respective cancer types when used to treat advanced metastatic disease[125-128]. There may therefore be a difference in the vascular biology of early microscopic tumours that render them insensitive to anti-angiogenic treatment, for example, an increased reliance on alternative modes of vascularization including vessel co-option[26].

The reasons the above failures in HCC are unclear[95]. The resistance mechanisms of anti-angiogenic therapies are generally thought to be broadly applicable in a variety of disease settings (Figure 1.3), however the resistance mechanisms that apply to sorafenib in HCC in particular are poorly understood. EMT signaling has been implicated in sorafenib resistance in a few studies of HCC and RCC tumour models[87,88]. Additional mechanisms that have been identified in pre-clinical studies but have yet to be validated in patients include enhancement of tumour cell EGFR signaling[129,130], increased autophagy[131] and enhanced survival of hypoxic tumour cells[132]. The success of sorafenib in advanced HCC suggests that angiogenesis is an important target, but a lack of efficacy in micrometastatic HCC[124] highlights an incomplete understanding of the vascular biology of the disease.

**Table 1.2** Completed phase III clinical trials for hepatocellular carcinoma treatment.

| Agent | Company | Setting | Year | Trial name | Overall survival (OS; months) | | Reference |
|---|---|---|---|---|---|---|---|
| | | | | | Experimental Arm | Standard of care arm | |
| Sorafenib | Bayer/Onyx | 1st line | 2007 | SHARP | 10.7 | 7.9 (placebo) | [117] |
| Sorafenib | Bayer/Onyx | 1st line | 2007 | Asia-Pacific | 6.5 | 4.5 (placebo) | [118] |
| Sunitinib | Pfizer | 1st line | 2011 | SUN1170 | 8.1 | 10.0 (sorafenib) | [133] |
| Erlotinib (+sorafenib) | Genentech | 1st line | 2012 | SEARCH | 9.5 | 8.5 (sorafenib+ placebo) | [94] |
| Brivanib | Bristol-Myers Squibb | 2nd line | 2011 | BRISK-PS | 9.4 | 8.2 (placebo) | [134] |
| | | 1st line | 2012 | BRISK-FL | 9.5 | 9.9 (sorafenib) | [93] |
| Linifanib | AbbVie | 1st line | 2014 | - | 9.1 | 9.8 (sorafenib) | [135] |
| Everolimus | Novartis | 2nd line | 2014 | EVOLVE-1 | 7.6 | 7.3 (placebo) | [136] |
| Ramucirumab | Eli Lilly | 2nd line | 2014 | REACH | 10.5 | 9.1 (placebo) | [137] |
| Sorafenib | Bayer/Onyx | Adju-vant | 2014 | STORM | 33.4 month recurrence-free survival | 33.8 month recurrence-free survival (placebo) | [124] |

## ORTHOTOPIC XENOGRAFT MODEL OF REVERSIBLE SORAFENIB RESISTANCE

### Hep3B-hCG model of HCC and reversible sorafenib resistance

In order to study the effects of sorafenib therapy in a preclinical model, the Kerbel laboratory previously developed an orthotopic human xenograft mouse model of HCC. In this model an 'hCG'-tagged human HCC cell line is injected into the left liver lobe of severe combined immunodeficient (SCID) mice (Tang et al.[138]; Figure 1.5A-B). The human Hep3B cell line was transfected with cDNA for the beta subunit of human chorionic gonadotropin (βhCG), and the same procedure was adapted to other cell lines[138]. βhCG is constitutively expressed and secreted by the transduced cancer cells and thus serves as a secretable, non-invasive surrogate protein biomarker of tumour burden that can be readily detected in the urine of mice (levels are normalized to urine creatinine)[138,139]. The 'hCG' system was previously

developed by Vogelstein and colleagues as a means to indirectly measure tumour burden in orthotopically xenografted tumours which could not be directly measured by calipers without sacrifice of the animal[139].

Using this model, Hep3B-hCG tumour-bearing mice were treated daily by gavage with oral sorafenib. Mice were given periodic breaks in treatment (typically lasting 3-5 days, starting at 10% average body weight loss, and ending at recovery to >5% weight loss) to permit recovery from drug-related host toxicity and to permit prolonged treatment[140] (Figure 1.5). This dosing schema resembles how sorafenib is often administered in patients in clinical settings, in which dose reductions and dose interruptions are commonly used to control toxicity[141,142]. In mice, sorafenib administered at 30 and 60 mg/kg/day markedly suppressed tumour growth, a response that continued for approximately 40 days. Following this initial period of tumour stasis, urinary hCG levels rebounded while mice were on therapy, therefore demonstrating a classic pattern of acquired drug resistance.

Conventionally, classical drug resistance is generally considered to be a heritable and permanent phenotype: it would persist following rechallenge with the same or similar drug, and it is transferrable to new hosts during experimental re-implantation into new hosts[143]. To study the nature of the resistance phenotype in the HCC xenografts, ostensibly sorafenib-resistant Hep3B-hCG tumour cell variants were adapted to culture, expanded in *vitro* for a few weeks and then were subsequently re-transplanted into tumour-naïve SCID mice. Surprisingly, the resultant tumours in secondary hosts were completely resensitized to sorafenib retreatment[140] (Figure 1.5D-E). The resistance phenotype was therefore 'lost' prior to re-treatment. The basis of this reversible resistance was unknown, but a few possible explanations include drug withdrawal and host-dependent effects.

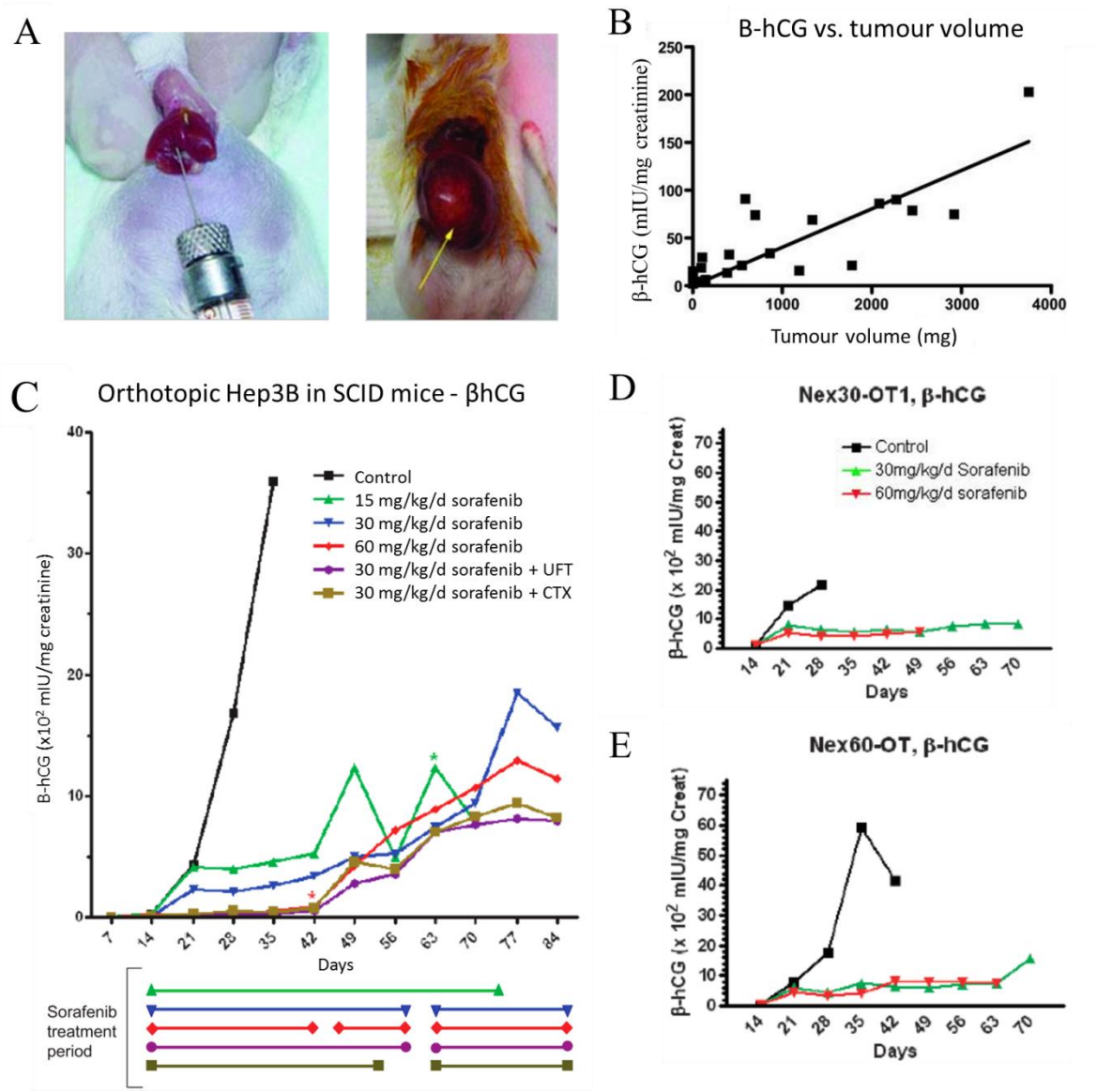


**Figure 1.5** Reversible sorafenib resistance in an orthotopic model of hepatocellular carcinoma. **A**. Intrahepatic implantation of Hep3B cells transfected with cDNA encoding β human chorionic gonadotropic hormone (β-hCG) into severe combined immunodeficient (SCID) mice leads to the development of locally advanced HCCs (yellow arrow). **B**. Urine β-hCG measurement normalized to urinary creatinine correlates well with tumour mass, thus serving as a tool to monitor tumour burden. **C**. Established orthotopic Hep3B-hCG tumours are initially highly responsive to oral sorafenib at 15, 30 or 60 mg/kg per day, relative to vehicle control-treated mice. After approximately 1 month of therapy, tumours rebounded thus demonstrating acquired resistance. Mice were dosed according to toxicity (schedules indicated by bars). **D-E**. In vivo resistant cancer cells were adapted to culture and re-implanted into new hosts. Growth of two resistant variants derived from mice originally treated with 30 mg/kg (D) or 60 mg/kg (E) sorafenib became resensitized to treatment indicating a reversible resistance phenotype. β-hCG from pooled urine samples (n=5/group) are shown. Data from Tang T. et al.[138,140].

## Other preclinical examples of reversible TKI resistance

Two other xenograft studies of anti-angiogenic TKI resistance reached similar conclusions. In one study, sorafenib-resistant subcutaneous RCC tumours grown in mice were homogenized and immediately injected into naïve hosts (without an intervening period of growth in vitro)[37]. Re-implanted tumours were sensitive to sorafenib treatment. Microarray analysis indicated that expression of approximately 75% of genes in the tumour that were altered during resistance reverted to baseline/untreated levels upon re-implantation[37]. In a second study, metastatic RCC tumour fragments from a patient who progressed on sunitinib were grafted subcutaneously into nude mice[89]. Xenografts became sensitive to sunitinib treatment. In this case a reversible histological phenotype was observed: the metastasis appeared to have undergone EMT, whereas the xenograft reverted back to an epithelial-like clear cell tumour. Taken together, these studies suggest that resistance to anti-angiogenic TKIs may be reversible.

## Reversible drug resistance phenotype in patients

Across different malignancies, drug resistance is commonly attributed to a selection of cancer cell clones that have acquired genetic changes over the course of therapy[143,144], or to overgrowth of cancer cells with pre-existing genetic alterations that render particular drugs ineffective[145]. Some agents, particularly those that target a driving oncogenic pathway in the cancer cell population, such as inhibitors of EGFR (erlotinib) or BCR-ABL1 (imatinib) often result in stable, heritable resistance. Moreover acquired point mutations in the targeted kinase domains of EGFR and ABL are frequently identified in biopsies of relapsed patients[146,147]. It is a fundamental notion in oncology that drug resistance is a permanent phenotype that equates to treatment failure. Thus, convention in the clinic is to permanently terminate a therapy at the point of disease progression (as a surrogate indicator of drug resistance) and switch to a new therapy.

Reversible or unstable drug resistance is not a well-recognized or well-understood concept. Recent evidence suggests that reversible resistance may apply to anti-angiogenic agents in patients, in addition to anti-angiogenic TKIs in animal tumour models. For instance, RCC patients frequently receive sequential lines of anti-angiogenic TKIs because despite the inhibition of similar targets including VEGFRs and PDGFRs, these TKIs have anti-tumour efficacy in sequence and display a lack of true cross-resistance[148,149]. Additionally, re-administering ("rechallenging") RCC patients with the same drug, either sunitinib or sorafenib, after they had apparently developed resistance to that agent has been shown to result in high rates of disease stabilization or objective tumour responses[150,151]. This typically occurs in patients after a "drug holiday", or an intervening period between initial and rechallenge treatment. Finally, a recently completed phase III clinical trial showed that after patients had progressed on a combination of chemotherapy plus bevacizumab, continuing bevacizumab while switching the chemotherapy regimens led to a prolongation in overall survival by 1.4 months compared to only switching the chemotherapy[152]. Thus, treatment of bevacizumab beyond progression (beyond the point of supposed 'drug resistance') led to a survival benefit albeit modest in magnitude.

As discussed in detail in Chapter 4, this phenomenon may extend beyond anti-angiogenic therapies. Similar resistance phenotypes may occur with chemotherapy, hormonal therapy and molecular-targeted therapy treatment. An important implication of reversible resistance is that drug sensitivity may be restored in a patient, thereby improving the activity of anticancer drugs after they have presumed to be inactive.

## PURPOSE AND OUTLINE OF WORK

Since resistance to anti-angiogenic therapy may not be absolute[153], the current understanding of resistance mechanisms and how best to treat resistant disease is incomplete. Developing strategies to delay the onset of resistance or treat resistant disease is of critical importance. This is particularly true of HCC since it is such a deadly disease with no therapeutic options after progression on sorafenib; thus the possibility of re-treatment with the same or similar therapy is of major value.

For the present studies, an orthotopic xenograft model of HCC was chosen as a model system to recapitulate human HCC as well as the pattern of clinical anti-angiogenic response and subsequent acquired resistance to therapy. The purpose of this work is to investigate the mechanisms of reversible sorafenib resistance[140] and to determine and optimize therapeutic strategies that can evade or delay sorafenib resistance in HCC.

My original hypothesis at the initiation of this project was that continuous drug treatment was necessary to maintain the sorafenib-resistance phenotype, and as such tumour cells may become resensitized following adaptation to *in vitro* culture prior to treatment in new hosts[140,154,155] or during a drug holiday[150,151,156]. Alternatively or additionally, host factors and/or the tumour stroma might be important for sorafenib resistance[84,90,157]. Moreover, these changes might be reversible since unlike the host, cancer cells may acquire permanent genetic alterations or mutations during tumour progression. Since the majority of proposed resistance mechanisms to anti-angiogenic agents implicate re-induction of angiogenesis during resistance (rebound angiogenesis)[60], the implicated host factors would likely contribute in some way to a resurgence of the vasculature by way of angiogenesis. Finally, due to the reversible nature of the resistance phenotype, resistance may be evaded or delayed by intermittent dosing of sorafenib or incorporating therapy breaks.

The mechanisms implicated in my studies relating to sorafenib resistance proved more complicated. The development of resistance appeared to be multifactorial. Moreover, a resurgence of angiogenesis did not appear to contribute to resistance. In Chapter 2, a potential pharmacokinetic cause of drug resistance is described – that of a decline in the plasma levels of sorafenib over time which correlates with the onset of tumour progression. In Chapter 3, a switch from tumour dependence on perfusion by angiogenic vessels to perfusion by vessel co-option was identified, which was facilitated by increased invasiveness of the cancer cells into the liver parenchyma. Moreover, some of the associated molecular and structural changes were apparently reversed by stopping therapy, suggesting that there is a reversible component to these resistance mechanisms. In Chapter 4, a literature review on reversible anti-cancer drug resistance is presented, which highlights the many examples in patients in which a reversible or unstable drug resistance phenotype occurs. As summarized in Chapter 5, the finding of reversible sorafenib resistance in HCC may have broader implications for other oncologic indications and drugs including anti-angiogenic agents, and raises the notion of a new type of 'anti-vascular' (rather than anti-angiogenic) therapy: preventing or targeting vessel co-option.

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# Chapter 2. Effects of Declining Sorafenib Levels and Dose on Acquired Reversible Resistance and Toxicity in Hepatocellular Carcinoma

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Attributions: Technical assistance with animal work was provided by Christina Lee. Contributions to experimental design were made by Christina Lee and Shan Man. Eric Chen and his laboratory performed the pharmacokinetic analysis of plasma and tissue samples.

## ABSTRACT

Acquired resistance is a major limitation of hepatocellular carcinoma (HCC) treatment with the tyrosine kinase inhibitor (TKI) sorafenib. Recent findings suggest that resistance to sorafenib may have a reversible phenotype. Additionally, loss of responsiveness has been proposed to be due to a gradual decrease in sorafenib plasma levels in patients. Here, the possible mechanisms underlying reversible sorafenib resistance were investigated using a Hep3B-hCG orthotopic human xenograft model of locally advanced HCC. Tissue and plasma sorafenib and metabolite levels, downstream anti-tumour targets and toxicity were assessed during standard and dose-escalated sorafenib treatment. Drug levels were found to decline significantly over time in mice treated with 30 mg/kg/day sorafenib coinciding with the onset of resistance but a greater magnitude of change was observed in tissues compared to plasma. Skin rash also correlated with drug levels and tended to decrease in severity over time. Drug level changes appeared to be partially tumour-dependent involving induction of tumoural CYP3A4 metabolism, with host pre-treatment alone unable to generate resistance. Escalation from 30 to 60 mg/kg/day sorafenib improved anti-tumour efficacy but worsened survival due to excessive body weight loss. Microvessel density was inhibited by sorafenib treatment but remained stable over time and dose increase. In conclusion tumour CYP3A4 induction by sorafenib is a novel mechanism to account for variability in systemic drug levels, however declining systemic sorafenib levels may only be a minor resistance mechanism. Escalating the dose may be an effective treatment strategy, provided toxicity can be controlled.

## INTRODUCTION

The oral anti-angiogenic tyrosine kinase inhibitor (TKI) sorafenib remains the only approved systemic treatment for advanced hepatocellular carcinoma (HCC). Sorafenib is an inhibitor of Raf serine/threonine kinases and receptor tyrosine kinases associated with vascular endothelial growth factor receptors (VEGFR) 2 and 3, platelet derived growth factor receptor (PDGFR) β, Flt-3 and c-Kit[1] and other signaling pathways including STAT3[2]. In two randomized phase III trials in advanced HCC patients, sorafenib treatment improved the time to progression and extended overall survival by 2.8[3] and 2.3 months[4] compared to placebo. These benefits are unfortunately modest, with upfront (innate/intrinsic) and acquired (evasive/secondary) drug resistance being major contributing factors. Toxicity is also an issue leading to a high rate of dose reductions and treatment interruptions in patients[5,6].

Proposed mechanisms for resistance, based primarily on preclinical studies, are diverse and numerous. Typically the host tumour microenvironment has been shown to be involved in an adaptive response to anti-angiogenic treatment leading, for instance, to re-induction of tumour vascularization and adaptation to, or escape from, tumour hypoxia – which can lead to hypoxia-inducible factor (HIF)-1-dependent activation of genes involved in angiogenesis, glycolytic metabolism, oxygen consumption, migration and invasion[7,8]. Cancer cells intrinsically may drive resistance, and most of the proposed sorafenib resistance mechanisms in HCC suggest this[9].

There has also been the suggestion that 'pharmacokinetic resistance' could develop to TKIs[10,11]. Standard doses of oral sorafenib leads to high (~50%) inter-individual variability in drug exposure[12-14] suggesting that some patients may be under-dosed. The absence of certain toxicities that are associated with improved clinical outcomes (e.g. hand-foot skin reaction (HFSR)[15,16]) may also indicate under-exposure and dosing[17,18]. Inadequate target inhibition and tumour-regrowth could result, appearing as intrinsic resistance. Additionally, sorafenib plasma levels have been shown to decline over time. In a

study of 15 HCC patients, sorafenib exposure was found to decrease significantly from one month of treatment (area under the curve, AUC 60.3 mg·h/L) to the time of disease progression (33.2 mg·h/L; p=0.007)[11]. This trend was later reported in other malignancies[13,19,20]. Such drug level changes could lead to reduced toxicities and acquired resistance and might be managed simply by longterm dosage adjustments. The cause of declining sorafenib exposure is unclear.

Additionally, resistance to TKIs occasionally presents as a reversible rather than permanent phenotype[21]. Patients with renal cell carcinoma (RCC) who had progressed on/acquired resistance to sorafenib[22] or sunitinib[23] have been reported to respond to rechallenge with the same agents. It has been argued that clinical trials evaluating the strategy of switching to another anti-angiogenic TKI after 'resistance' has developed should contain an arm evaluating continuation of treatment with the same TKI since this strategy is often efficacious[24]. Reversible sorafenib resistance has been modeled preclinically in HCC using mice bearing intrahepatic human xenografts of Hep3B-hCG cells[25]. These mice demonstrate initial marked sensitivity to sorafenib which is followed by tumour rebound after one month, based on levels of the secreted urinary protein tumour biomarker βhCG (β human chorionic gonadotropic hormone) and tumour weight changes[25]. Once ostensibly resistant tumour cells were adapted to culture and re-implanted into new hosts, the resultant tumours were completely re-sensitized to retreatment[25]. Reversible resistance may be a common but under-appreciated phenomenon but how it relates to other resistance mechanisms is unclear.

In the present study, mechanisms underlying reversible resistance to sorafenib were investigated in the Hep3B-hCG model of HCC. The hypothesis that sorafenib pharmacokinetics might mediate the resistance mechanism was explored.

## MATERIALS AND METHODS

### Orthotopic mouse model

Athymic nude mice (*nu/nu*; Harlan) were used for experiments assessing sorafenib concentrations. CB17 severe combined immunodeficient (SCID) mice (Charles River) were used for sorafenib 'pre-conditioning' and in-house bred yellow fluorescent protein (YFP) expressing CB17 SCID mice were used for transfer of resistant phenotype experiments (breeding pairs a gift from Dr. Janusz Rak). Mice were male aged 6-8 weeks. Animal procedures were in accordance with institutional animal care and maintenance guidelines.

The Hep3B-hCG model of HCC was described previously[26]. Briefly, HCCs were established by injecting 1-2x$10^6$ Hep3B-hCG cells/10 µL volume into the left liver lobe of anesthetized mice[26]. Individual mouse urine βhCG levels (henceforth, "hCG") normalized to creatinine served as a noninvasive surrogate biomarker for tumour burden[26]. The Hep3B-hCG cell line was authenticated by STR DNA analysis (Genetica DNA Laboratories) and was found to be mycoplasma-free (Lonza).

In resistance transfer experiments, in vivo resistant 2-3mm tumor fragments were implanted into tumor-naïve SCID mice by subcutaneous implantation into the right hind flank, or by orthotopic suturing onto the liver. Sorafenib or vehicle treatment resumed after 2 days or approximately one month, respectively.

### Cell culture

Cultures were grown in high-glucose Dulbecco's modified Eagle medium (Hyclone) containing 5% heat-inactivated fetal bovine serum (FBS; Hyclone) in a humidified incubator at 37ºC/5% $CO_2$. For analysis of resistant variants adapted to culture from sorafenib-resistant SCID mice, resistant cells were

grown in the absence of sorafenib and compared to parental Hep3B-hCG cells. Proliferation was assessed by MTS assay following 5 days of treatment with various doses of sorafenib. 5000 cells of each variant were seeded onto 6-well plates and clones were imaged after two weeks. To evaluate ERK phosphorylation, subconfluent cells were serum-starved 24h then treated in the presence of DMSO or 5μM sorafenib in the presence or absence of (20 ng/ml; R&D Systems) prior to protein lysis. Cellular invasion was assessed by the Boyden chamber assay by allowing cells to invade through a layer of 2mg/ml Matrigel (BD Biosciences) toward 5% FBS-DMEM chemoattractant over a 72h period.

## Sorafenib dosing and toxicity monitoring

Sorafenib tosylate was obtained from Bayer with the assistance of Dr. Dennis Healy and was prepared according to manufacturer's recommendations. Unless otherwise indicated, oral gavage treatment of 30 mg/kg/day sorafenib or vehicle control began at hCG>0 (tumour diameter ~1-2 mm). Tumour response was defined as hCG stabilization (decline from prior assessment and/or hCG<200 mIU/mg) and a progression defined as hCG>200 mIU/mg. In cases where mice progressed early, treatment was continued to confirm tumour response. Dose was switched to 60 mg/kg on treatment day 29 approximating the average time of tumour progression.

Therapy was temporarily stopped at 10% average body weight loss in SCID mice and re-initiated following recovery to ≤5% (3-5 day therapy breaks) mimicking the way toxicity is frequently managed clinically[5]. For sorafenib pre-conditioning, tumour-free SCID mice were treated with sorafenib or vehicle as per tumour-bearing mice for 45 or 65 days, were given a 2-day washout period then were orthotopically implanted with parental Hep3B-hCG cells and randomized. At 5 days after surgery, treatment was resumed according to the schedule of the group experiencing the most toxicity. Continuous daily dosing was possible for athymic *nu/nu* mice, which tolerate TKIs better than SCID mice[25]. Skin toxicity was monitored biweekly by briefly anesthetizing and photographing *nu/nu* mice. Rash was

graded as ‘rash’, ‘no rash’, or ‘resolved rash’. Resolved rash appeared as clear skin or rashes that diminished significantly in area (>~95%) and redness from prior assessment.

## Plasma and tissue sampling

Flash-frozen tumour and heparin-plasma samples (by cardiac puncture) were initially obtained 24h after dosing ($c_{trough}$). In subsequent experiments, mice were fasted for 3h, dosed, and heparin-plasma was obtained from the retro-orbital sinus 3h later to achieve maximal drug/metabolite concentrations ($t_{max}$ 1-3 h in mice[27,28]). Plasma samples were taken from different tumour-free and HCC mice 2 weeks pre-treatment, days 1, 4, 7 and 11. Serial biweekly samples were obtained from a different set of mice for weeks 2+. Weighed flash-frozen liver and tumour and formalin-fixed tumour samples were obtained at sacrifice (3h after dosing) on day 14 (sensitive) and at endpoint (control, resistance and dose escalation phases). Plasma or homogenized tissue samples (100 µL volume) were analyzed by HPLC tandem mass spectrometry (HPLC-MS/MS) to determine sorafenib and estimate N-oxide metabolite levels as previously described[29,30].

## Protein analysis

Protein expression in cell culture, liver and tumour lysates was analyzed by Western blot (40µg protein/well) using the following antibodies: phospho-STAT3 (Tyr705; 4113), STAT3 (4904), mouse/human CYP3A4 (13384), phospho-ERK (Thr202/Tyr204; 4376), ERK (4696), phospho-PDGFRβ (Tyr751, 4549) and PDGFRβ (3169; all Cell Signaling Technology), and βactin (A5441; Sigma-Aldrich). An ELISA for mouse VEGFR2 phosphorylation (P-VEGFR2) was developed using phospho-Tyr and mouse VEGFR2 antibodies (MAB4431, BAF644, BAM1676) and recombinant mouse VEGFR2 as an internal standard (443-KD-050; all R&D Systems).

### Immunohistochemistry

Formalin fixed, paraffin-embedded tumour sections were immunostained using the following reagents: For microvessel density (MVD; CD34+ and CD31+ vessels), 1:150 CD34 (LS-C47878; LifeSpan Biosciences), 1:100 CD31 (sc-1506; Santa Cruz Biotechnology), Cy3- and Alexa488-conjugated secondary antibodies (Jackson Immunoresearch) and DAPI (Invitrogen); for cell proliferation (human Ki67), 1:1000 Ki67 antibody (VP-K451; Vector Laboratories), LSAB+ and DAB+ kits (Dako) and hematoxylin (Surgipath). ~20 images/section were obtained (n=4-9/group) at 100X (Ki67) or 200X (MVD). CD34+ or CD31+ microvessel counts and Ki67 stain were normalized to nuclear stain using ImageJ (v.1.46r) software.

### Statistical analysis

Experimental group differences were evaluated by Student's T-test or one-way ANOVA with Bonferroni's correction for multiple comparisons. The effects of tumour presence and time on drug concentrations were assessed by two-way ANOVA. Correlations between hCG and drug concentrations were determined by linear regression. Survival was evaluated by log-rank test using GraphPad Prism (v.4.00). Data is presented as the mean and standard error of the mean. Significance level was set at 0.05.

## RESULTS

### Plasma sorafenib declines over time

Variants from *in vivo* sorafenib (30 mg/kg/day, day 86 of treatment) resistant Hep3B-hCG tumours were first assessed *in vitro* for resistant properties. The resistant variants were no more proliferative, invasive or able to survive as clones than the parental Hep3B-hCG cells (Figure 2.1A-D),

but rather tended to be less aggressive than parental cells. Resistant variants and parental cells were sensitive to MAPK inhibition by sorafenib in vitro (Figure 2.1D). Thus, no indication of resistance was observed *in vitro*. The resistant phenotype was also lost by transferring resistant tumour fragments or *in vitro* chronically sorafenib-exposed variants into tumour-naïve hosts (Figure 2.1F-G and Figure 3.7, Chapter 3), suggesting that host treatment is important for resistance. Host-wide pharmacokinetic changes are associated with disease progression in patients[11,13,19,20], therefore this was investigated as a mediator of the reversible sorafenib-resistant phenotype in mice.

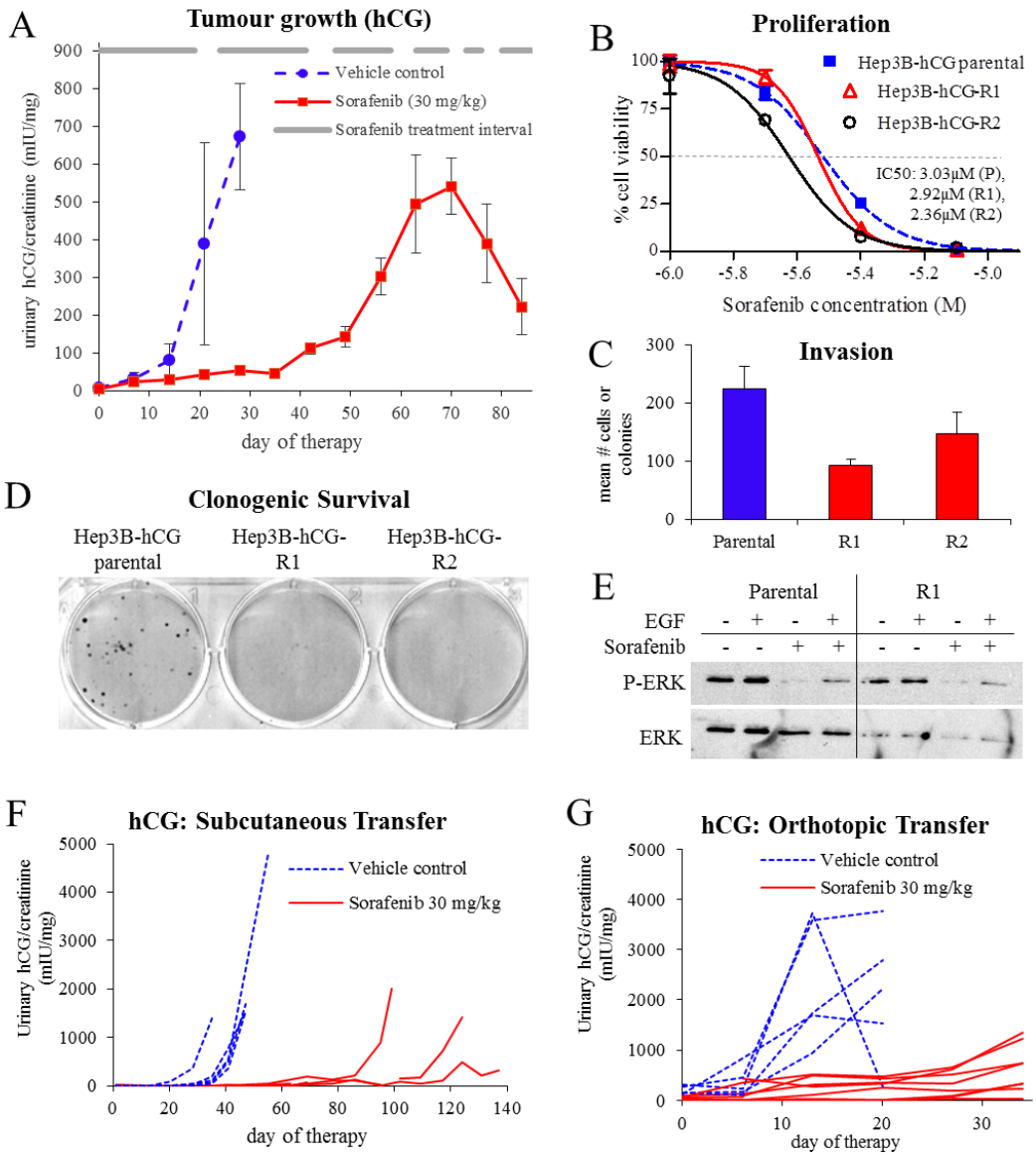


**Figure 2.1** Loss of sorafenib resistance in vitro and during transfer of resistant tumour fragments. **A**. SCID mice bearing Hep3B-hCG intrahepatic xenografts treated with 30 mg/kg/day sorafenib (treatment schedule indicated) show a pattern of acquired resistance (resistance beginning ~day 45) indicated by levels urinary hCG secreted by tumour cells (n=5). The resistant properties of in vitro adapted resistant tumour cells were assessed. **B**. Parental cells and resistant variants Hep3B-hCG-R1 and R2 had similar proliferation rates with the 50% inhibitory concentrations shown. **C**. Resistant variants tended to be less invasive (ANOVA p=0.07; n=3). **D**. Resistant variants had poorer clonogenic survival than parental cells. **E**. Parental cells and Hep3B-hCG-R1 were sensitive to phosphorylated (P) ERK inhibition by sorafenib (5μM) in the presence or absence of EGF or DMSO (24h treatment). **F**. Transfer of tumour fragments into tumour-naïve hosts by subcutaneous implantation resulted in tumour sensitivity when therapy was resumed 2 days later (n=5-6). **G**. Orthotopic suturing of tumour fragments and treatment resumption after tumours became established also led to a loss of resistance (n=5, 8).

Drug levels in tumour and plasma samples were analyzed from athymic *nu/nu* mice bearing Hep3B-hCG xenografts treated daily with 30 mg/kg sorafenib. Samples were obtained from sensitive (treatment day 13, n=6), pre-progression (day 33, n=6), and acquired resistant mice (days 47-88, n=8; Figure 2.2A). Trough ($c_{trough}$, 24h) measurements were taken to estimate steady state levels (shown to correlate with TKI treatment outcome[31,32]), and to overcome the obstacle of obtaining multiple timed plasma samples. As per HCC patients[11], a significant reduction in plasma sorafenib concentration was observed in mice that had acquired resistance relative to responsive mice (70.6% decline, $p<0.05$; ANOVA $p=0.02$; Figure 2.2B). Tumour sorafenib levels tended to decline from $c_{trough}$ measurement but this was not statistically significant (ANOVA $p=0.31$; Figure 2.2C). Thus, declining systemic drug levels correlated with resistance in mice with HCC.

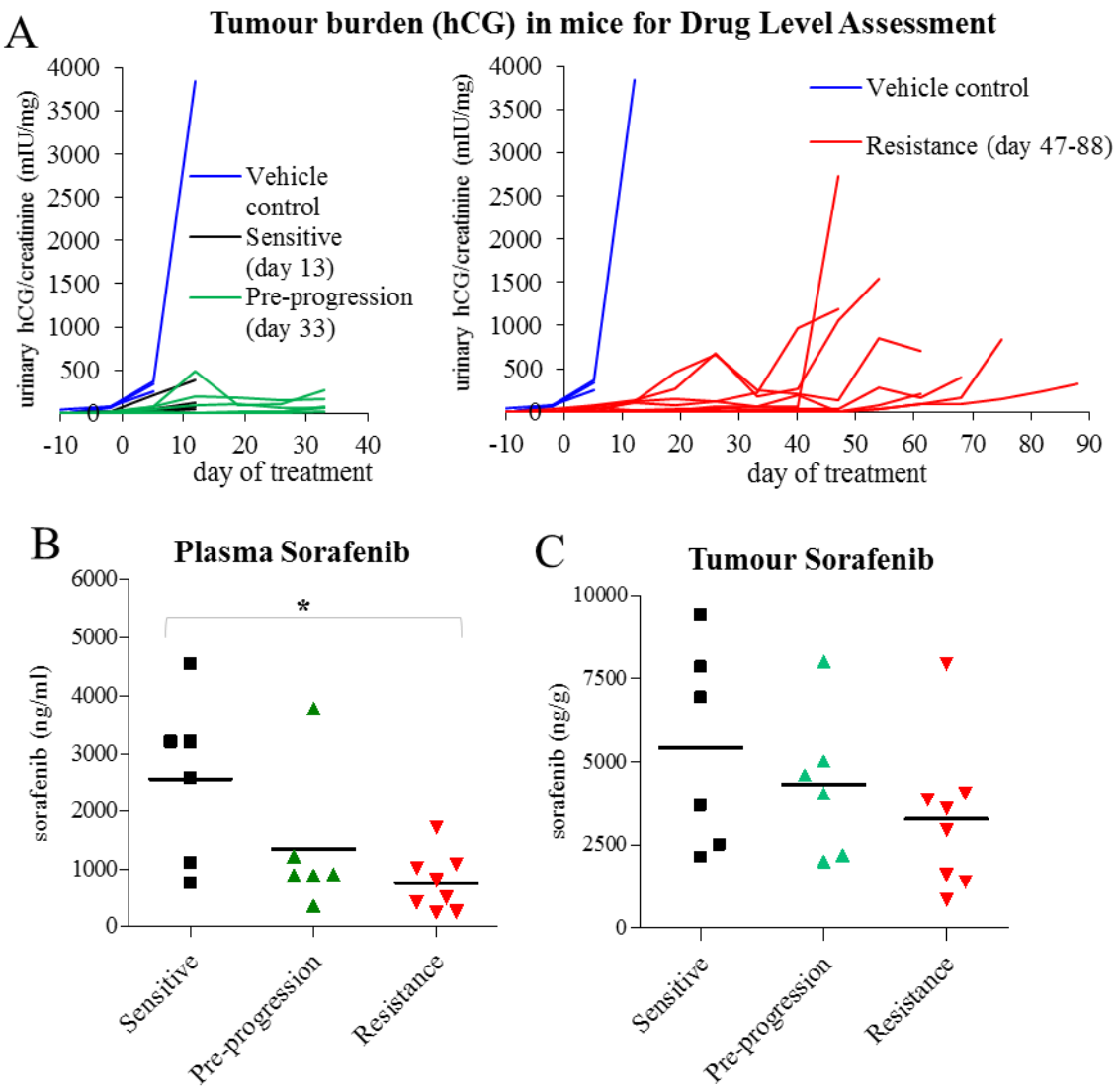


**Figure 2.2** Decline in plasma sorafenib levels is associated with resistance in HCC. **A**. Athymic nu/nu mice bearing intrahepatic xenografts of Hep3B-hCG cells eventually acquire resistance to daily 30 mg/kg/day sorafenib treatment as demonstrated by urinary hCG levels normalized to creatinine. Plasma and tumours were obtained 24h after dosing for drug level assessment from drug sensitive, pre-progression and acquired resistant mice (days 13, 33 and 47-88 respectively; n=6-8). **B**. Sorafenib plasma concentration was found to decline significantly from sensitive to resistant time points (ANOVA p=0.02, *p<0.05), indicating a potential pharmacokinetic resistance mechanism. **C**. Corresponding tumour concentrations of sorafenib also tended to decline but not significantly (ANOVA p=0.31).

## The presence of tumour intensifies declining sorafenib levels

Both host and tumour factors could conceivably contribute to the observed decline in sorafenib concentration in the plasma. In the host, 5% of sorafenib is oxidated by hepatic p450 enzyme CYP3A4[33] and 15% glucuronidated by UGT1A9[34] contributing to fecal and urinary elimination, respectively. The oxidated N-oxide metabolite is pharmacologically active but more hydrophobic than the parent compound and represents the dominant circulating metabolite in humans (9-16% of total sorafenib)[34] and pharmacological induction of CYP3A4 has been shown to decrease systemic sorafenib concentrations[33,35]. In contrast, drug efflux transporters P-glycoprotein and ABCG2 are thought to play minor roles in sorafenib pharmacokinetics[36,37]. In the tumour, drugs may accumulate in cancer cells (such as in acidic lysosomes for sunitinib)[10] or cancer cells may themselves express drug metabolizing enzymes[38] thereby influencing exposure levels. Thus, concentrations of sorafenib and its major metabolite were determined in the presence and absence of tumour to explore the mechanism of sorafenib decline.

Tumour-free and Hep3B-hCG tumour-bearing (HCC) mice were treated daily with sorafenib (30 mg/kg) and after 29 days half of mice were switched to 60 mg/kg, and plasma was sampled 3h after dosing to maximize drug concentrations. Peak plasma sorafenib was achieved days 4 to 7 in mice treated with 30 mg/kg sorafenib (Figure 2.3A) which then declined in both HCC ($R^2$=0.72, p=0.008) and tumour-free mice ($R^2$=0.81, p=0.006), however total sorafenib levels tended to be higher in tumour-free mouse plasma (Figure 2.3B). Both time (p<0.0001) and tumour presence (p=0.02) were significantly associated with plasma levels by two-way ANOVA. Tumour presence also significantly impacted %N-oxide levels (p=0.006 vs. time p=0.11) as %N-oxide peaked by day 11 in tumour-bearing mice only, but thereafter remained marginally higher than in tumour-free mice. The decline in sorafenib concentrations from sensitive (day 14) to resistance (endpoint) phases were more striking in HCC mouse tissues: a 52.1% (p=0.02) and 31.7% drop (p=0.002) was observed in tumours and in livers, respectively, while a non-

significant 19.4% decline was observed in tumour-free livers ($p=0.23$; Figure 2.3C). Tissue levels of %sorafenib N-oxide did not significantly change ($p>0.05$; Figure 2.3C).

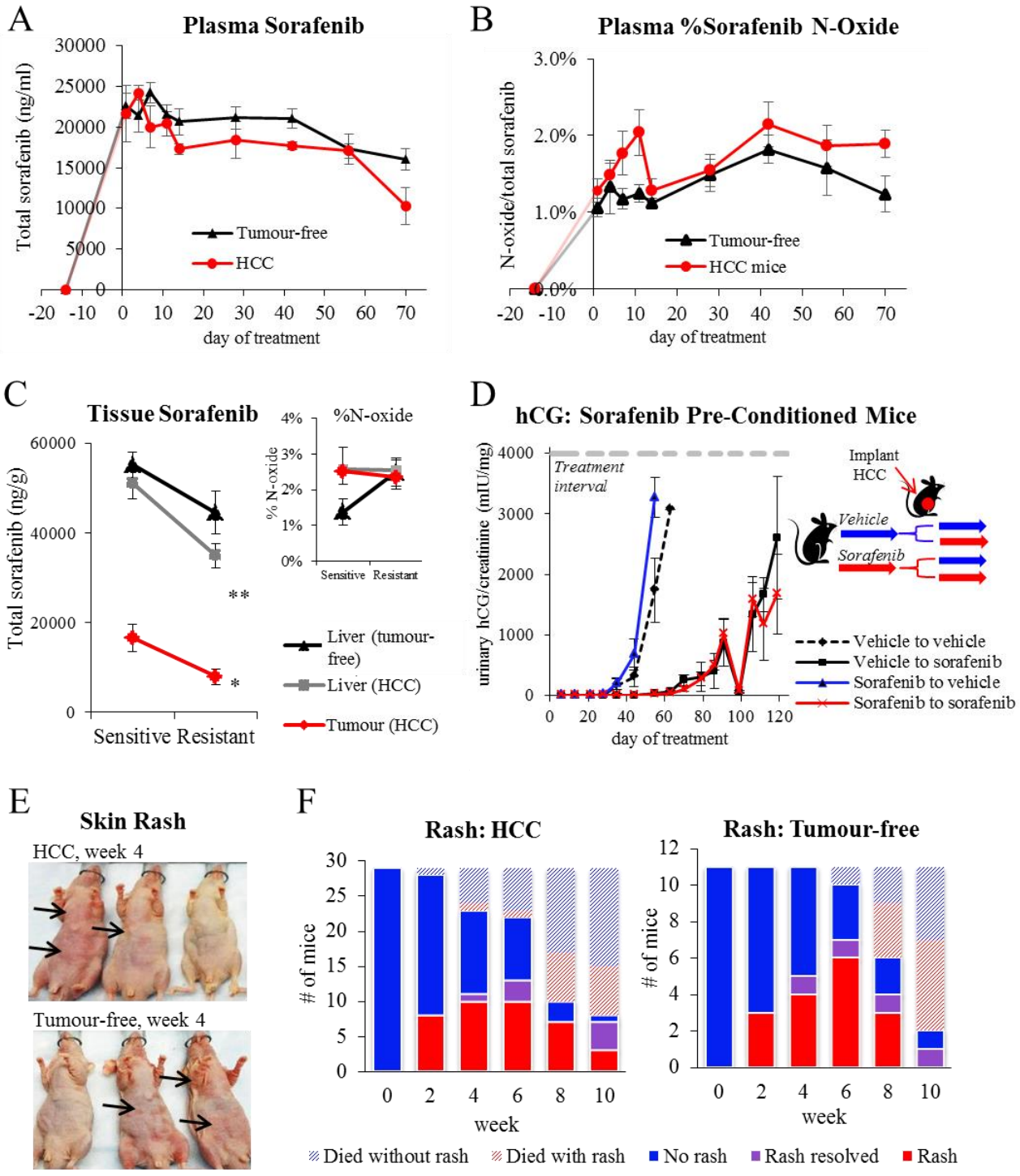
A
Plasma Sorafenib
Total sorafenib (ng/ml)
30000
25000
20000
15000
10000
5000
0
-20 -10 0 10 20 30 40 50 60 70
day of treatment
Tumour-free
HCC
B
Plasma %Sorafenib N-Oxide
N-oxide/total sorafenib
3.0%
2.0%
1.0%
0.0%
-20 -10 0 10 20 30 40 50 60 70
day of treatment
Tumour-free
HCC mice
C
Tissue Sorafenib
Total sorafenib (ng/g)
60000
40000
20000
0
Sensitive Resistant
**
*
%N-oxide
% N-oxide
4%
3%
2%
1%
0%
Sensitive Resistant
Liver (tumour-free)
Liver (HCC)
Tumour (HCC)
D
hCG: Sorafenib Pre-Conditioned Mice
urinary hCG/creatinine (mIU/mg)
4000
3000
2000
1000
0
Treatment interval
0 20 40 60 80 100 120
day of treatment
Implant HCC
Vehicle
Sorafenib
Vehicle to vehicle
Vehicle to sorafenib
Sorafenib to vehicle
Sorafenib to sorafenib
E
Skin Rash
HCC, week 4
Tumour-free, week 4
F
Rash: HCC
# of mice
30
25
20
15
10
5
0
0 2 4 6 8 10
week
Rash: Tumour-free
# of mice
12
10
8
6
4
2
0
0 2 4 6 8 10
week
Died without rash
Died with rash
No rash
Rash resolved
Rash

**Figure 2.3** Tumour impact on drug concentrations and resistance, and variation in rash development. **A**. Total sorafenib plasma concentrations sampled 3h after dosing tended to decline in tumour-free ($R^2$=0.81 days 7-70, p=0.006) and Hep3B-hCG-bearing (HCC) mouse plasma ($R^2$=0.72 days 4-70, p=0.008) but drug levels were generally higher in tumour-free mice. The tumour (p=0.02) and time (p<0.0001) significantly impacted drug levels (two-way ANOVA). **B**. The %N-oxide metabolite/total sorafenib was marginally higher in HCC mouse plasma (two-way ANOVA, p=0.006 tumour, p=0.11 for time) and peaked in HCC mice day 11 (T-test p=0.04). **C**. Sorafenib concentrations declined significantly from sensitive to resistant phases in the liver (p=0.002; n=10-11) and tumour (p=0.02; n=8-9) of HCC mice but not in tumour-free mouse livers (p=0.23; n=6). Tissue levels of the %sorafenib N-oxide (right) did not significantly change (p>0.05) in either group but tended to increase in tumor-free livers (p=0.07). **D**. SCID mice pre-conditioned for 45 days with 30 mg/kg/day sorafenib and subsequently implanted orthotopically with parental tumours, developed resistance at the same rate as vehicle pre-conditioned mice (n=4-5). **E**. Skin rash toxicity frequently developed in tumour-free and HCC athymic nu/nu mice (arrow) when mice were sorafenib-responsive. **F**. Rash eventually improved weeks 4+ in mice, potentially relating to drug level decline.

## The host effect on resistance is minimal

To determine the extent to which host-induced changes contributed to resistance, tumour-free SCID mice were pre-conditioned with sorafenib for 45 or 65 days prior to tumour implantation, corresponding to early and late onset of resistance (data shown for 45 day pre-conditioning, Figure 2.3D). SCID mice were given brief toxicity-associated therapy breaks allowing extended treatment times[25]. Sorafenib pre-conditioning for either duration worsened weight loss but did not accelerate the onset of resistance relative to vehicle pre-conditioning (Figure 2.3D and data not shown). Thus, the presence of HCC tumour worsened the gradual decline in sorafenib but the host effect alone appeared minimal as host treatment was insufficient to generate resistance.

## Incidence of mouse skin toxicity parallels decline of sorafenib levels

Skin toxicities, including rash and HSFR, are the most commonly reported adverse events in sorafenib-treated patients[5]. Skin toxicity has been correlated with drug exposures[18,39] and improved

clinical outcomes[15,40] but has paradoxically been reported to decrease in severity over time[13,41,42]. 74% of HCC (n=43) and 75% of tumour-free mice (n=20) developed a non-irritated red skin on the ventral skin surface, ranging from small spots to nearly the entire surface (Figure 2.3E). Rash was a treatment effect since stopping therapy caused complete rash resolution (and weight gain) in 7/7 mice within 1-2 weeks (data not shown). While weight loss was slow and progressive in sorafenib (30 mg/kg/day) treated mice, rash initially developed weeks 2 to 6 but tended to resolve beginning week 4 (Figure 2.3F). 42% of HCC mice treated >6 weeks (n=12) and 44% tumour-free mice (n=9) showed rash improvement. Although delayed in time, this result may reflect declining tissue drug levels.

## Tumour-mediated sorafenib metabolism correlates with declining drug levels

The effect of the tumour on drug levels was further explored. Negative correlations were observed between sorafenib concentrations and tumour burden/hCG. This relationship was strongest in the tumour ($R^2$=0.25, p=0.04) and approached significance in the liver ($R^2$=0.15, p=0.08) and the plasma ($R^2$=0.05, p=0.08; Figure 2.4A). Contrastingly, plasma %N-oxide correlated positively with hCG ($R^2$=0.28, p<0.0001). Thus, local tumour drug levels and plasma %N-oxide associated well with the degree of tumour burden, but associations with plasma drug concentrations and tumour responses were weak.

Tumour and liver lysates were analyzed to determine expression levels of CYP3A4 enzyme. CYP3A4 protein levels were found to increase during sorafenib treatment in tumours (individual and pooled lysates) compared to vehicle-treated controls (Figure 2.4B-C), and were further induced in dose-escalated tumours. In contrast, CYP3A4 remained stable in HCC and tumour-free livers (Figure 2.4C). Of note, baseline levels of CYP3A4 were lower than in the liver, but induced CYP3A4 did not exceed liver enzyme levels. While dose escalation increased plasma sorafenib concentrations (Figure 2.4D), it also induced the %N-oxide metabolite, which appeared to relate to CYP3A4 levels (Figure 2.4E). Thus, auto-

induction of sorafenib metabolism may explain, at least in part, the tumour contribution to systemic sorafenib level decline.

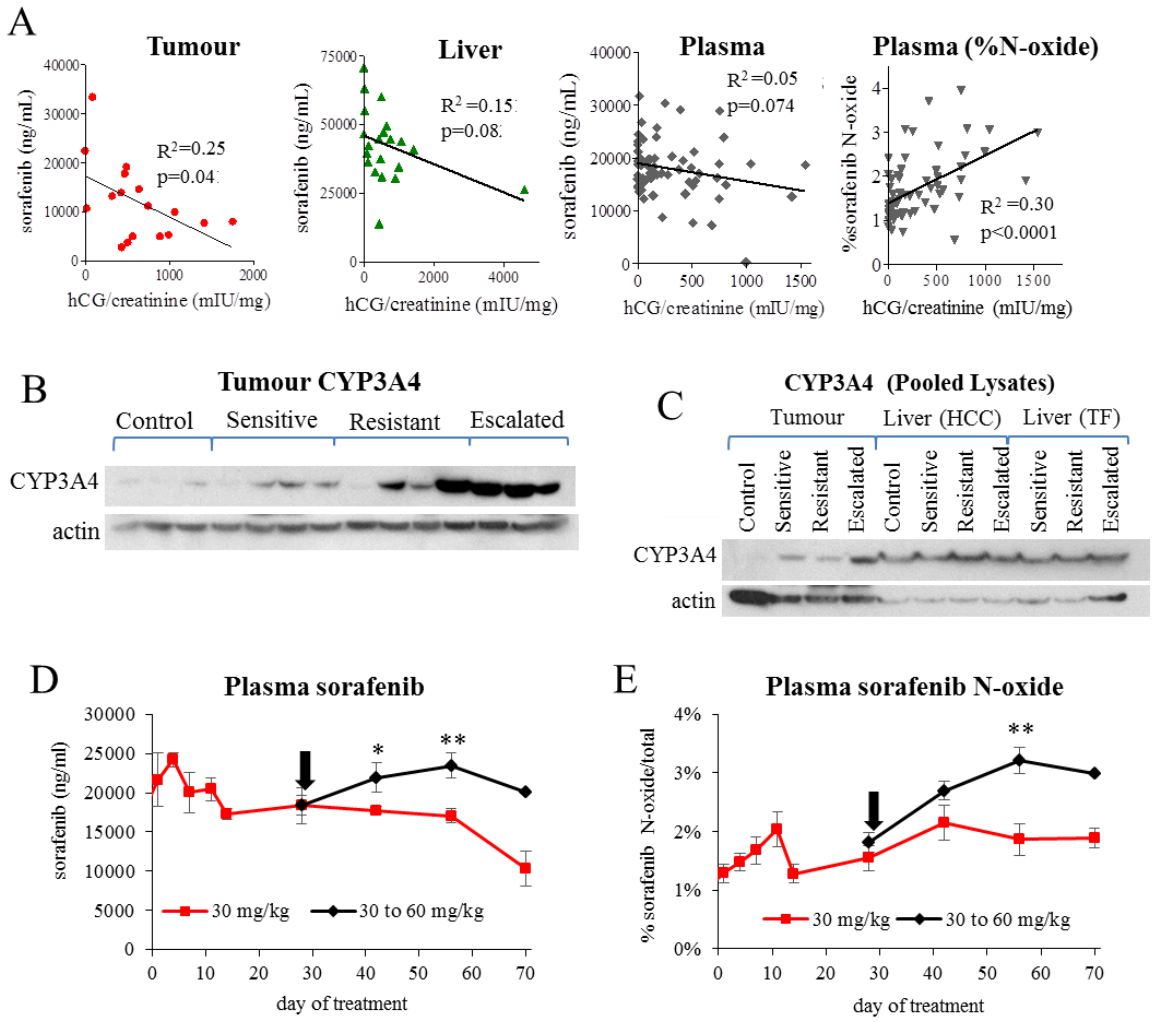


**Figure 2.4** Sorafenib levels are associated with tumoural drug metabolism. **A**. Tumour burden (hCG) in 30 mg/kg sorafenib-treated mice (all time points for plasma; sensitive and resistance time points for tissues) correlated significantly with tumour total sorafenib concentrations ($R^2$=0.25, p=0.04) and plasma %N-oxide ($R^2$=0.28, p<0.0001) suggesting a link with drug metabolism. Correlations for total sorafenib approached significance in the liver ($R^2$=0.15, p=0.08) and plasma ($R^2$=0.05, p=0.07). **B**. CYP3A4 protein in individual tumours was induced by 30 mg/kg sorafenib treatment and re-induced by escalation to 60 mg/kg. **C**. Analysis of pooled lysates confirmed induction of CYP3A4 in tumour but not in livers (controls n=4, HCC n=9-10, tumour-free n=5-6). **D**. Dose escalation increased sorafenib plasma concentrations and **E**. the %N-oxide metabolite in HCC mice, consistent with CYP3A4 expression (n=10-12; *p<0.05, **p<0.01 vs. 30 mg/kg). Arrow= switch to 60 mg/kg/day sorafenib.

**Sorafenib dose escalation inhibits tumour growth at the expense of increased toxicity**

When the dose of sorafenib was doubled to 60 mg/kg on day 29 (beginning at the time of $c_{trough}$ decline Figure 2B and average hCG progression) plasma levels increased by only 23.6% from pre-escalation levels. This escalation strategy appeared effective in correcting drug level decline since plasma concentrations were restored to early time-points (Figure 2.4D) and concentrations significantly increased in the livers ($p=0.009$) and tumours ($p=0.04$) of HCC mice though this did not correspond with significant %N-oxide increases except, interestingly, in tumour-free livers (Figure 2.5A).

Dose escalation was evaluated for its ability to treat or prevent resistant disease. By day 29, progressive disease occurred in 17/43 mice. In all mice dose escalation inhibited hCG (Figure 2.5B) in addition to tumour plus liver mass at endpoint ($p<0.01$; data not shown). This dose escalation strategy was not however effective in reversing resistance since disease continued to progress at a rate similar to non-escalated mice. Dose escalation also resulted in excessive weight loss (Figure 2.5C), which was the primary reason for termination (20% weight loss endpoint), leading to significantly decreased median survival (log rank $p=0.002$; Figure 2.5D). Tumour presence had no significant impact on weight loss or survival ($p>0.05$ and data not shown), therefore drug toxicity appeared to be a critical factor in survival. Dose escalation worsened skin rash by week 6, then 58% of HCC mice (n=12) and 50% of tumour-free mice (n=8) experienced rash improvement (Figure 2.5E).

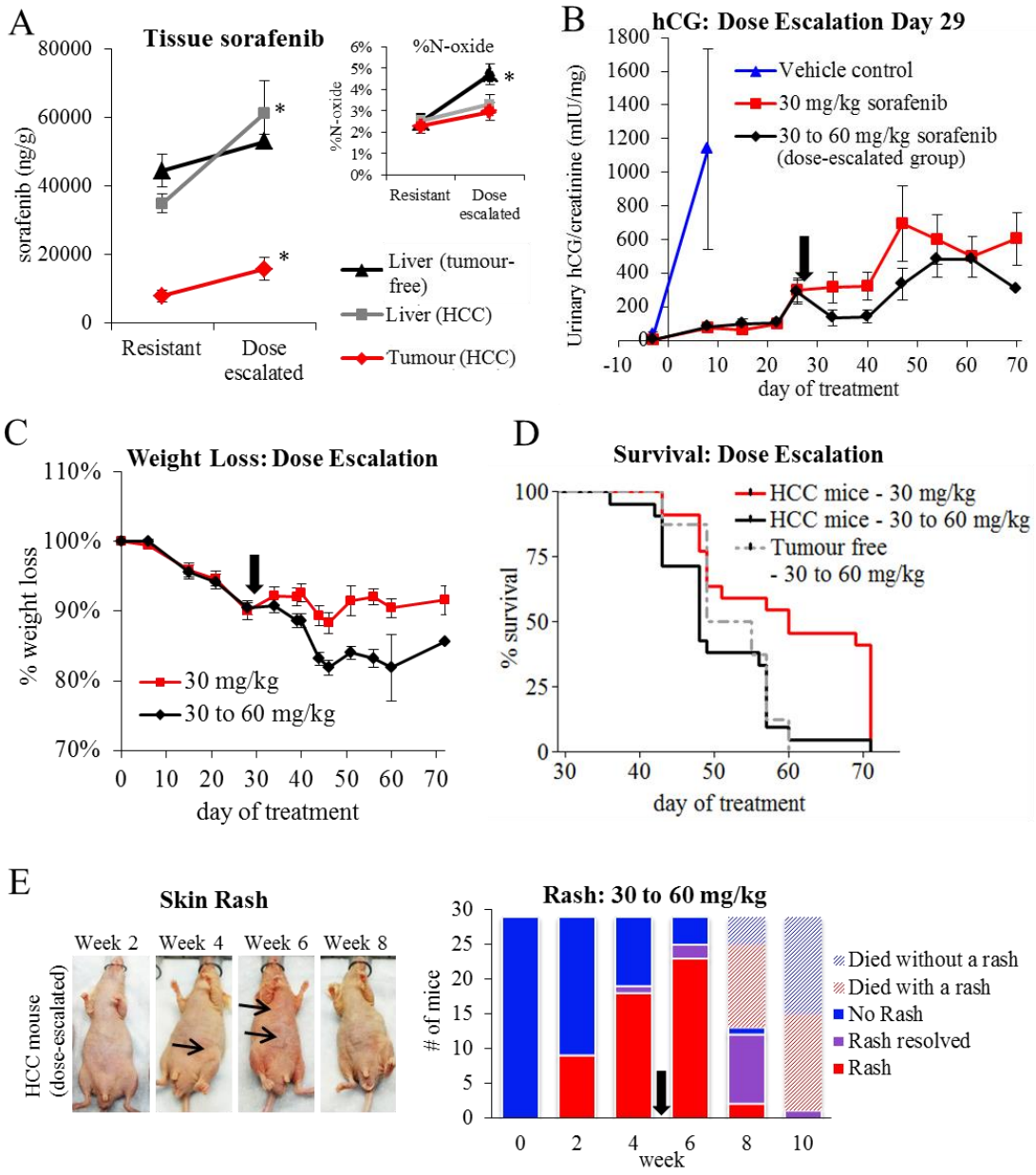


**Figure 2.5** Impact of dose escalation. **A**. Dose-escalating sorafenib to 60 mg/kg/day significantly increased drug levels in the livers (p=0.009; n=11, 9) and tumours (p=0.04; n=8-9) of HCC mice, but not in tumour-free livers (p=0.16; n=5-6) *p<0.05. The %sorafenib N-oxide (right) tended to increase in the livers (p=0.15) and tumours (p=0.20) of HCC mice, but significantly increased in tumour-free livers (p=0.06). **B**. Escalating the dose at disease progression slowed tumour growth (n=5 control, 21-22 sorafenib). **C**. Sorafenib-treated mice lost weight which was accelerated by dose escalation. **D**. Mice maintained on 30 mg/kg sorafenib had superior survival (median 60 days) compared to switching to 60 mg/kg/day (48 days, log-rank p=0.002; n=21-22). Mice without tumours did not exhibit prolonged survival during dose-escalation (median survival 52 days; p=0.60). **E**. Skin rash worsened by week 6 after dose escalation then tended to improve. Arrow=switch to 60 mg/kg/day.

## Inhibition of angiogenesis is not associated with drug level changes

The possibility that circulating drug concentrations were suboptimal for target inhibition and anti-tumour effect was investigated. No inhibition in Raf (ERK), VEGFR2 or PDGFRβ signaling by sorafenib treatment was detected in tumour lysates however (Figure S2.6A-B). Only P-STAT3, a potential down-stream mediator of sorafenib activity[2], was inhibited by initial sorafenib treatment, which subsequently increased during resistance in livers from both HCC and tumour-free mice (Figure 2.6C), potentially related to changes in local sorafenib levels. Similar findings were observed in tumours although initial P-STAT3 inhibition during sorafenib treatment was not observed. Escalation to 60 mg/kg/day sorafenib had little impact on P-STAT3 despite increases in tissue drug concentrations (Figure 2.6C).

Of those tested, the predominant anti-tumour mechanism for sorafenib treatment appeared to be anti-angiogenesis. Sorafenib-treated tumours were less hemorrhagic than controls, but dose-escalated tumours were smaller and appeared more white/necrotic (Figure 2.6D). Sorafenib treatment significantly inhibited microvessel density ($p<0.001$ vs. controls, ANOVA $p<0.0001$) but during resistance there was no evidence of resumption of angiogenesis (Figure 2.6E). Likewise, hypoxia-responsive carbonic anhydrase IX (CAIX) protein remained elevated throughout treatment (data not shown). In contrast, tumour cell proliferation was not significantly affected by treatment (ANOVA $p=0.08$; Figure 2.6E), except for minor inhibition during dose escalation (T-test $p=0.01$ vs. resistance). Altogether, systemic drug levels appeared sufficient for inhibiting angiogenesis therefore factors other than an increase in microvessel density must be responsible for causing HCC tumour progression.

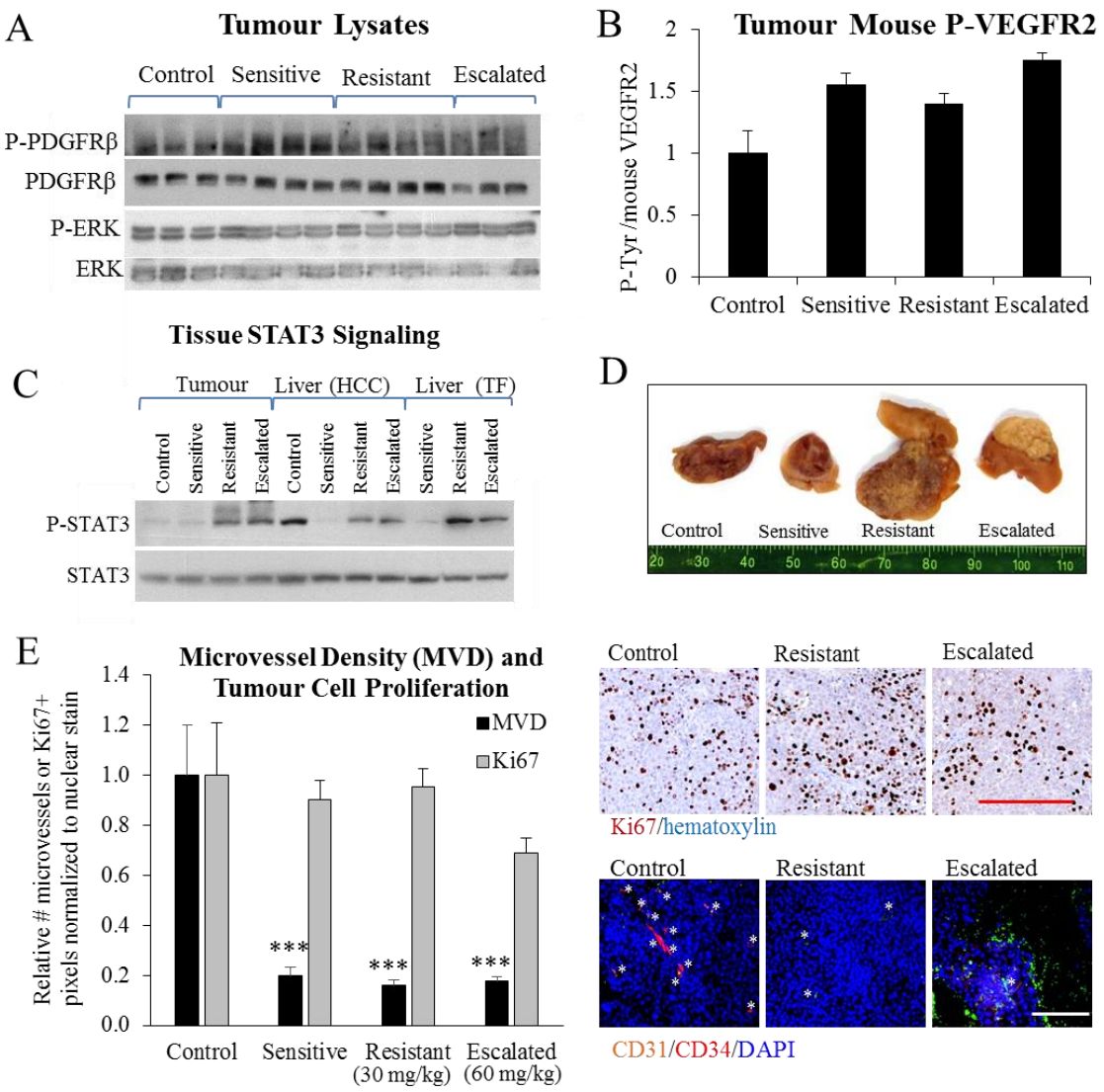


**Figure 2.6** Effects of sorafenib on cell signaling and anti-tumour activity. **A**. Sorafenib treatment was not associated with inhibition of phosphorylated (P) PDGFRβ or ERK in tumour lysates derived from individual mice. **B**. No inhibition of VEGFR2 phosphorylation was observed by sorafenib treatment based on ELISA analysis of tumour lysates (n=6-8/group). **C**. Sorafenib treatment inhibited phosphorylation of downstream target STAT3 (P-STAT3) in livers (pooled lysates). P-STAT3 increased in tumours and livers of resistance phase HCC and tumour-free mice, but remained high in dose-escalated tissues, indicating some correlation with local drug levels. **D**. Fixed tumour cross-sections show hemorrhagic control tumours and increasingly pale treated tumours. Dose escalated tumours were small and appeared necrotic/white. **E**. Tumour cell proliferation (human Ki67 immunostaining) did not significantly change during treatment (ANOVA p=0.08) but tended to decrease during dose escalation (T-test p=0.01 vs. resistant). Tumour microvessel density (CD31 (green) and CD34 (red) vessel counts normalized to DAPI (blue)) was significantly inhibited throughout 30 and 60 mg/kg sorafenib treatment (ANOVA p<0.0001; ***p<0.001), showing little association with drug levels. Representative images are shown at right. Bar=500 µm.

## DISCUSSION

In recent clinical studies sorafenib exposure was reported to decline by up to 50% at the time of disease progression in small groups of patients with HCC[11], melanoma[20] and other solid tumours[13,19]. A similar observation has been made in GIST patients treated with the TKI imatinib[43], but as with sorafenib, the underlying causes are unknown. Here, clinical observations were confirmed in mice bearing HCC xenografts and a possible mechanism was identified. However, the involvement of this phenomenon in reversible resistance appears complex.

### Mechanism of reduced drug exposure over time

In patients pharmacological induction of CYP3A4 by anti-epileptic drugs or rifampin significantly decreased sorafenib exposure[33,35] suggesting a key role for this metabolic pathway. CYP3A4 inhibition by ketoconazole or midazolam had little effect on exposure except for reducing metabolite levels[44,45], which is expected given that only 5% of an oral dose is metabolized by CYP3A4[33]. Auto-induction of drug metabolism in the tumour has largely been an under-appreciated contributor to resistance[38]. Here, *in vivo* tumour induction of CYP3A4 by sorafenib was found to be a possible contributor to declining drug levels. This result is consistent with findings that TKIs gefitinib and sunitinib induced expression of p450 enzyme CYP1A1 in various cancer cell lines[46,47]. A potential pathway mediating these effects of sorafenib may be via nuclear pregnane X receptor (PXR)-mediated transcriptional activation of CYP3A4, which is directly activated by bound ligand (ie. sorafenib) [48]. Despite the key role for hepatic metabolism, no liver induction of CYP3A4 was observed during treatment, in agreement with observations by others[27,28]. Conceivably, only tumour cell CYP3A4 levels were induced by sorafenib because hepatic enzyme levels are constitutively expressed at very high levels which are sufficient for adequate drug biotransformation.

Tumoural CYP3A4 induction does not explain the weaker sorafenib decline in tumour-free mice. It is possible that additional factors, such as drug-binding plasma proteins levels, decreased intestinal absorption, and involvement of other metabolic pathways (e.g. UGT1A9) contribute to sorafenib level changes in the host, and in patients overall. Due to the high tumour:body mass ratio of mice in xenograft studies, the importance of a tumour-dependent mechanism may have been exaggerated in these studies, further highlighting the need for follow-up investigation.

The sorafenib dose used here (30 mg/kg) is considered low following conversion to a human equivalent dose[49]. Plasma concentrations (~20,000mg/ml) were in the range of mouse studies[27] but higher than in patients (<10,000 ng/mL with 400 mg b.i.d.[12]). In humans the N-oxidation pathway is more pronounced than in mice[28], which is validated by the N-oxide levels observed here (1-3% vs. 9-16% in humans[34]). While higher %N-oxide might be expected from CYP3A4 induction, it cannot be ruled out that generated metabolite was cleared too rapidly for direct quantification. Alternatively, the observed metabolite levels may indicate a minor impact of the tumour on drug levels. Indeed P-STAT3 and rash patterns (potential pharmacodynamic markers) were similar regardless of tumour presence, but the relationship between pharmacokinetics and pharmacodynamics can be complex.

## Dose escalation as a therapeutic strategy

In selected populations of RCC and HCC patients, slowly ramping up the dose of sorafenib[42] or increasing the dose at progression[50,51] have demonstrated tolerability and anti-tumour activity. Dose escalating may also serve to re-establish adequate exposure levels[11,13,19,20]. Here, dose was doubled concurrently for all mice regardless of weight loss status. This did not prove to be effective: anti-tumour activity increased but tolerability was poor. Lower-than-predicted plasma drug levels also occurred as is common in dose-escalated patients, which may indicate saturated drug absorption[12,14] or poor drug solubility at higher doses[28].

Based on the data presented here, therapeutic plasma drug monitoring could underrepresent drug levels changes within the tissues, which could mean missed opportunities to optimize the dosage. Dose escalating according to toxicity is an alternative. Skin rash in mice recapitulated observations of rash and HSFR in TKI-treated patients. Rash developed at a high rate for up to 6 weeks correlating with early treatment response[15,16] and tended to improve in ~50% of cases mimicking the reported decreasing severity of skin toxicity[13,41,42]. Rash improvement may therefore be directly related to declining drug levels as suggested by correlations with AUC in patients[13,20]. The uncoupling between patterns of weight loss and rash appears consistent with clinical findings and may relate to higher unabsorbed drug concentrations in the gut[20]. To manage excessive weight loss, dose interruptions should be incorporated, which may also help re-sensitize the tumour[21]. Optimized strategies that combine dose increases with brief therapy breaks may hold at least some anti-tumour activity while prolonging survival. Extensions in PFS and OS have been achieved in a retrospective study using a similar strategy with sunitinib in RCC[52].

## Declining sorafenib levels as a resistance mechanism

At first glance systemic drug level changes appear to correlate with resistance and hence provide a possible mechanism for the reversible resistant phenotype that cannot be propagated to new hosts[25]. Tissue sorafenib levels related to tumour progression and P-STAT signaling. Interaction between the host and tumour cells was also found to be critical for resistance. However, the lack of microvessel density change during treatment suggests that drug levels remained sufficient for longterm angiogenesis inhibition, which is based on the assumption that microvessel density is directly correlated to the anti-tumour effect. However was cannot be confirmed due to the lack of observed change in VEGFR2 phosphorylation, which could have arisen from technical issues (e.g. cross-reactivity with human tumour cells and/or poor sensitivity for endothelial VEGFR2 expression). It is possible that declining sorafenib levels is a minor or a contributing factor to resistance. Likewise, dose escalation slowed tumour growth

but did not directly prevent tumour progression or effectively treat resistant disease. This might explain why the benefits of sorafenib dose escalation are often transient[19,42].

In conclusion, sorafenib levels declined over time in mice but its impact on resistance is unclear. Escalating the dose may be an effective strategy however more tolerable regimens are needed, particularly for HCC patients with impaired liver function. A relationship was also observed between skin rash in 'nude' mice and drug levels. Given the frequency of rash as a side effect of many biologic anti-cancer agents, these results could be extended to study the impact of rash as a potential biomarker of drug efficacy. While drug resistance remains a complex issue, individualizing treatment regimens with toxicity-guided approaches has potential to enhance the activity of currently available TKIs for cancer treatment.

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# Chapter 3. Co-option of Liver Vessels and Not Sprouting Angiogenesis Drives Acquired Sorafenib Resistance in Hepatocellular Carcinoma

A modified form of this chapter has been accepted for publication pending minor revisions. Authors: Kuczynski, E.A., Yin, M., Bar-Zion, A., Lee, C.R., Butz, H., Man, S., Daley, F., Vermeulen, P.B., Yousef, G.M., Foster, F.S., Reynolds, A.R., Kerbel, R.S.

Attributions: Technical assistance with animal work was provided by Christina Lee. Contributions to experimental design were made by Shan Man. Henriett Butz and George Yousef performed microRNA sequencing and RT-PCR analysis. Frances Daley and Andrew Reynolds developed the co-option staining methodology. Peter Vermeulen and George Yousef performed histopathological analysis of tissue specimens. Melissa Yin, Avinoam Bar-Zion and Stuart Foster were involved in ultrasound experiment design, image acquisition, processing and analysis.

## ABSTRACT

The anti-angiogenic agent sorafenib is standard of care for advanced hepatocellular carcinoma (HCC), but acquired resistance limits its efficacy. An emerging theory to explain intrinsic resistance to anti-angiogenic drugs is 'vessel co-option'; i.e., the ability of tumours to hijack the existing vasculature in organs, thus limiting the need for sprouting angiogenesis. However, vessel co-option has not been evaluated as a potential mechanism for acquired resistance to antiangiogenic agents. We used an orthotopic xenograft model of HCC to study sorafenib resistance mechanisms. Histopathology, vessel perfusion assessed by ultrasound and miRNA sequencing were used to monitor changes in tumour and vascular biology. While sorafenib initially inhibited angiogenesis and stabilized tumour growth, no angiogenic rebound effect was observed during resistance, unless therapy was stopped. Instead, resistant tumours became more invasive, which facilitated the extensive incorporation of liver parenchyma and the co-option of liver-associated vessels. Supporting this, miRNA sequencing implicated pro-invasive signaling and epithelial-to-mesenchymal-like transition during the development of resistance, while functional imaging supported a shift from angiogenesis to vessel co-option in treated tumours. We propose vessel co-option as a mechanism of acquired resistance to anti-angiogenic therapy. This could have important implications for anti-angiogenic treatments including the therapeutic need to target vessel co-option in conjunction with VEGF.

## INTRODUCTION

Angiogenesis is considered to be particularly important in advanced hepatocellular carcinoma (HCC). The oral anti-angiogenic tyrosine kinase inhibitor (TKI) sorafenib is the only systemic treatment shown to demonstrate a survival benefit in advanced HCC[1,2]. Sorafenib inhibits angiogenesis by targeting pro-angiogenic growth factor receptors, namely vascular endothelial growth factor receptors (VEGFR) 1-3 and platelet-derived growth factor receptor (PDGFR) β but may also directly inhibit cell proliferation through the Raf/MAPK pathway[3]. 35-43% of HCC patients' tumours respond transiently to sorafenib treatment[1,2], but ultimately these patients acquire resistance and are taken off therapy.

One theory to explain resistance to angiogenesis inhibition is through activating VEGF-independent sprouting angiogenesis[4]. Compensatory pro-angiogenic ephrins, fibroblast growth factors (FGFs), angiopoietin-1 (Ang1), Ang2, interleukin (IL)-8, hepatocyte growth factor (HGF), PlGF, PDGF and VEGF-C or pro-inflammatory cytokines including G-CSF and IL-17, have each been shown to be up-regulated during development of resistance to anti-VEGF therapy leading potentially to angiogenic rebound and tumour re-perfusion[4,5]. This theory has yet to be clinically validated. For example, combined inhibition of VEGF and FGF has not proven effective in phase III clinical trials[6]. In studies that have utilized tumour perfusion imaging, contrast enhancement patterns suggestive of angiogenic re-induction occur in only some patients at the point of progression[7-9]. Analogous progression patterns in HCC have not been reported.

Another potential mechanism of resistance to anti-angiogenic therapy is vessel co-option, the recruitment of pre-existing vessels by the tumour. Vessel co-option has been reported in angiogenesis-inhibitor naïve tumour types including human colorectal carcinoma liver metastases[10] non-small cell lung carcinomas and lung metastases[11,12] and others[13]. However, its role during acquired resistance to anti-angiogenic therapy has not been established[13]. Deregulated signaling pathways within the cancer cell

population have also been proposed drivers of sorafenib resistance in HCC[14], but such work is limited primarily to *in vitro* studies.

Clinical experience with VEGF inhibitors suggests that resistance may take on a transient or unstable phenotype[15]. Rechallenging patients with sunitinib or sorafenib after progression on either drug may prolong progression-free survival[16,17] suggesting that TKI resistance could be reversible. Continuing VEGF inhibition therapy beyond progression (resistance) with the monoclonal antibody bevacizumab also led to survival benefits in a phase III trial in metastatic colorectal carcinoma patients[18] and recently an increase in progression-free survival in a phase III trial of HER2-negative metastatic breast cancer[19]. The basis of this reversible and unstable resistance phenotype is not clear.

To study the basis of acquired sorafenib resistance we employed an orthotopic HCC model which recapitulates initial clinical response and subsequent resistance. This resistance phenotype is reversible since it cannot be transferred to new hosts[20]. We used histology, molecular analysis and high resolution contrast-enhanced ultrasound imaging to elucidate possible resistance mechanisms operating *in vivo.* Our results implicate a switch to vessel co-option, facilitated by enhanced tumour cell invasion.

## METHODS

### Orthotopic mouse model of HCC

6-8 week-old male in-house bred CB17 severe combined immunodeficient (SCID) mice expressing yellow fluorescent protein (breeding pairs were a gift from Dr. Janusz Rak, McGill University) were used for tumour sampling and imaging studies. Female CB17 SCID mice (Charles River) were used for implantation of chronically drug-exposed resistant variants. The Hep3B-hCG HCC model was

previously described[21]. Briefly, Hep3B cells were transfected with cDNA encoding the β subunit of human chorionic gonadotropic hormone (βhCG). Individual mouse urine βhCG levels normalized to creatinine (henceforth, "hCG") is a non-invasive tumour burden biomarker. $10^6$ Hep3B-hCG cells/10μL volume into the left liver lobe. Hep3B-hCG cells were authenticated by STR DNA analysis (Genetica DNA Laboratories) and found to be mycoplasma-free (Lonza). Therapeutic response was monitored weekly by urine hCG measurement. Animal care and experimental procedures were performed in accordance with the Animal Care Committee and Comparative Research Department of Sunnybrook Research Institute.

## Sorafenib dosing

Sorafenib tosylate was obtained from Bayer with the assistance of Dr. Dennis Healy and was prepared according to manufacturer's recommendations. Gavage treatment of 30 mg/kg sorafenib or vehicle control began once hCG>0 (tumour diameter ~1-2mm). Therapy was temporarily stopped for 4 days at 10% average body weight loss from treatment initiation allowing weight recovery to ≤5% and treatment resumption, thus mimicking the clinical management of sorafenib toxicity[22]. Tumour sampling and imaging occurred after a minimum of 3 days dosing to avoid potential confounding effects of transient tumour flare.

## Experimental design

When >50% of mice had detectable urine hCG (~1 month post-implantation), mice were assigned to either a longitudinal ultrasound imaging study or a molecular/histological analysis study. All mice were randomized to vehicle or daily 30 mg/kg oral sorafenib treatment. 17 mice were assigned to one group for longitudinal ultrasound imaging. These mice had 55% greater mean hCG than the remaining mice such that these larger tumours were of sufficient volume for imaging. Remaining mice were randomized for

sacrifice at each at the following five treatment phases (n=6/group) 24h after dosing: vehicle Control (day 13 from start of treatment); sorafenib-Sensitive when mice were responsive to therapy (day 13); Early Resistance, defined as an individual disease progression (early hCG peak) to an hCG value of 400 mIU/mg (with hCG signal on an upward trend) or >200 mIU/mg for two consecutive weeks (day 33 (n=1), day 54 (n=3) and day 63 (n=2)); Late Resistance (day 76), hCG>200 mIU/mg for ≥4 consecutive weeks; and 'Stop' sorafenib therapy (day 76), in which treatment was permanently interrupted for 13 days following resistance development. Livers were partitioned for formalin-fixation and histochemical analysis with liver attached or carefully dissected and flash-frozen for molecular analysis.

One tumour variant derived from a mouse that acquired sorafenib resistance after 84 days was treated *in vitro* with fresh 5% FBS-containing Dulbecco's Modified Eagle's Medium with 1.5 μM sorafenib (selected empirically based on 50% growth inhibition over 5 days) or DMSO every two days for 35 days. $10^6$ control or sorafenib-treated cells were then implanted orthotopically into the livers of tumour-naïve mice. 30 mg/kg sorafenib or vehicle treatment was resumed after five days.

## Dynamic contrast enhanced ultrasound imaging (DCEUS)

*In vivo* imaging was performed on a high-frequency ultrasound system (Vevo®2100, VisualSonics Inc.). Vehicle (n=4) and sorafenib-treated mice (n=11) were imaged at day 0 and weeks 2, 5, 7 and 10 after starting treatment. Contrast enhanced images were collected using non-linear contrast imaging after a 50μL bolus intravenous injection of MicroMarker UCA. Global image analysis was completed offline using the VevoCQ contrast analysis software (Bracco S.p.A.). A ROI encompassing the whole tumour was drawn for each contrast injection cineloop to generate time-intensity curves (TIC) from which peak enhancement and wash-in-rates were determined. For high-resolution perfusion analysis a dedicated MATLAB® code (The Mathworks) was developed to correct for movement artefacts and to apply a 3x3 averaging pixel filter to allow for the detection of vessel distribution. Histograms of these

local perfusion parameters were generated in the normal liver, control and sorafenib-treated tumours. PE histograms included 30 bins ranging between 0 and 600 (e.g. bin 1= values 0-20).

## Histology and immunohistochemistry

Formalin-fixed, paraffin-embedded tumour sections were stained with hematoxylin and eosin (Surgipath) for analysis of necrosis, growth patterns and histopathological signs of invasion. The percent of tumour necrosis was calculated from whole-section scans threshholded to tumour, liver and viable total areas using ImageJ (v.1.46r). The following antibodies were used for immunostaining: 1:1000 anti-human Ki67 (VP-K451; Vector Laboratories), 1:500 anti-mouse/human cleaved caspase-3 antibody (9664; Cell Signaling), 1:150 anti-mouse CD34 (LS-C47878; LifeSpan Biosciences), 1:150 anti-mouse CD31 (sc-1506; Santa Cruz Biotechnology), 1:400 anti-mouse Ki67 (12202, Cell Signaling), 1:150 anti-human CAIX (AF2188; R&D Systems), FITC-conjugated anti-αSMA (F3777, Sigma-Aldrich) and 1:100 anti-human vimentin (M7020, Dako). Cy3- and Alexa488-conjugated secondary antibodies (Jackson Immunoresearch) with DAPI counterstain (Invitrogen) or LSAB+ Universal HRP System and DAB+ kits (Dako) with hematoxylin counterstain as appropriate.

## Co-option analysis

Tumour/liver sections were triple-stained sequentially using the following reagents: 1:30 rat anti-mouse CD31 (DIA-310, Dianova) detected with histofine HRP polymer (414311F, 2B Scientific) with TMB substrate (SK4400, Vector); 1:500 rabbit anti-human lamin A/C (ab108595, Abcam) detected with HRP/DAB (HRP Flex kit, Dako) and light hematoxylin and 1% aqueous eosin (both Surgipath). All vessels (1 section/tumour) were counted (29 in total; 1 ‘Stop’ tumour was omitted due to excessive size but appeared similar to group members) from images magnified at 200X.

Liver parenchyma-associated vessels that were considered part of the tumour and therefore quantified were sections of hepatocytes and sinusoidal/hepatocyte vessels 'invading' and oriented into the primary tumour (usually with hepatocyte plates perpendicular to the tumour front) or liver parenchyma completely surrounded by small peritumoural nodules. In the rare case where tumours grew as 2-3 distinct primary nodules, intercepting liver parenchyma vessels were not counted. Composite images were studied to plan the path of tumour rim as necessary. Vessels (1 vessel = 1 continuous CD31+ vessel or branch or large vessel continuing in and out of plane) were classified using the following criteria aided by a pathologist: 1) Tumour-embedded vessels were surrounded entirely by tumour cells; 2) hepatocyte-embedded vessels were directly adjacent to at least 1 hepatocyte or separated by a thin layer of mural cells; 3) connective tissue vessels were found within areas of hematoxylin-positive/lamin A/C-negative non-vessel mural cell fibroblasts; 4) central veins were large venules with structural integrity surrounded almost entirely by hepatocytes with a minimal stroma component; and 5) portal triads, 1 triad consisted of a vein/venule plus an artery/arteriole and a bile duct of simple cuboidal epithelium usually surrounded by connective tissue, with up to 1 component separated by tumour cells but in close proximity. Total counts were normalized to total tumour area (determined by counting lamin A/C-positive overlaid grid paper squares), and to viable tumour area.

## miRNA sequencing

cDNA libraries were prepared from tumours (n=2/group) using the TruSeq Small RNA Library Sample prep kit (RS-200-0012, Illumina) amplified by 11 PCR cycles and validated with the Bioanalyzer High Sensitivity DNA Kit (5067-4626, Agilent Technologies). Sequencing was performed on the Illumina Hi-Seq 2500 platform. Reads were aligned to a database of mature RNA sequences (mirBase 20) using novoalign v2.08.02. The number of reads uniquely mapping to each mature RNA sequence were counted and analyzed using EdgeR and limma analysis packages.

## Real-time PCR

Total RNA was extracted from tumour lysates (n=3/group) using miRNeasy kit (217004, Qiagen). 1 µg total RNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Life Technologies). Gene expression relative to HPRT and GAPDH was measured on the Viia 7 Real-Time PCR System (Life Technologies) by the ddCt method. The following primer sequences were used for real-time PCR analysis of EMT-related genes: VIM: forward (F) 5'-GACAATGCGTCTCTGGC ACGTCTT-3', reverse (R) 5'-TCCTGCAGGTTCTTGGCAGCCA-3'; ZEB1: F 5'-CGGCGCAATAACG TTACAA -3', R 5'-GGCAGGTCATCCTCTGGTACA-3'; ZEB2: 5'-TCTCCCATTCTGGTTCCTACA-3', R 5'-TTCTCATTCGGCCATTTACAG-3'; CDH1 F 5'-CACAGTCACTGACACCAACGATAA-3', R 5'-CTCAGGCACCTGACCCTTGTA-3'; CDH2: F 5'-TGGGAATCCGACGAATGG-3', R 5'-TGCAGAT CGGACCGGATACT-3'; SNAI1: F 5'-TCGGAAGCCTAACTACAGCGA-3', R 5'-AGATGAGCATTG GCAGCGAG-3', SNAI2: F 5'-AAGCATTTCAACGCCTCCAAA-3', R 5'-GGATCTCTGGTTGTGGTA TGACA-3'. The geometric means of GAPDH (GAPD; F 5'-GAAGGTGAAGGTCGGAGTC-3', R 5'-GAAGATGGTGATGGGATTTC-3') and HPRT (F 5'-TTGCTGACCTGCTGGATTAC-3', R 5'-TCTCCACCAATTACTTTTATGTCC-3') were used as endogenous controls.

## ELISA

Tumour protein lysates were analyzed by commercially available ELISA kits: mouse (m)VEGF-A (MMV00), human (h)VEGF-A (DVE00), mOPN (DY441), mSDF-1 (DY460), mG-CSF (DY414), mAng2 (MANG20), mPDGF-BB (MBB00), mPDGF-AB (MHD00), hbFGF (DFB50; all from R&D Systems), ERK and P-ERK (7050, 7177C; Cell Signaling) with recombinant human active ERK1 as a protein standard (E3452-46A; US Biological) and mAng1 (CSB-E0702m, Cusabio).

### Statistical analysis

Differences across experimental groups were evaluated by Student's T-test or by one-way ANOVA. Bonferroni's Multiple Comparison test assessed differences across groups. Global perfusion changes were tested by repeated measures one-way ANOVA. Differences between the frequencies of PE values at different time points were tested using two-sample T-tests. Data is presented as the mean and standard error of the mean. Significance level was set at $p<0.05$.

## RESULTS

### Orthotopically implanted HCC tumours develop resistance to sorafenib

Hep3B-hCG orthotopic HCC xenografts were simultaneously grown in two groups of SCID mice to characterize the phases of sorafenib-sensitive and sorafenib-resistant tumour growth. One group was used for molecular/histological analysis and another for contrast-enhanced ultrasound imaging. In the Hep3B-hCG model creatinine-normalized urine βhCG protein levels serve as a non-invasive biomarker for tumour burden[21]. Mice were treated with daily 30 mg/kg sorafenib on a discontinuous schedule in which therapy was temporarily stopped for 4 days upon 10% average weight loss allowing recovery from toxicity[20]. Such brief therapy breaks are also common in sorafenib-treated RCC[22] and HCC patients[1,2], thus reflecting well the clinical situation of toxicity management. Vehicle control tumours rapidly progressed, whereas sorafenib treatment initially stabilized tumour growth. We define "growth rate" =relative hCG increase per day. In Figure 3.1A, growth rate for 'Control' tumours were observed to be very high at 72.4 vs. -0.96 mIU/mg/day for sorafenib-treated tumours ($p<0.001$). This difference was reflected by a mean doubling time increase from 3 days (Control) to 9 days (sorafenib-sensitive). Sorafenib-sensitive tumours at two weeks of treatment are referred hereafter as 'Sensitive'. After 38 days,

tumours progressed more rapidly on sorafenib (hCG >200 mIU/mg, hCG rate change from 0.79 to 29.1 mIU/mg/day, $p<0.05$; mean doubling time 5 days), reflecting resistance to therapy[20]. Such tumours are referred to as 'Early Resistant' at the point of initial hCG progression, or "Late Resistant" after more prolonged treatment. Sustaining treatment led to irregular hCG changes, but stopping therapy for two weeks ('Stop' tumours) resulted in accelerated tumour growth relative to Late Resistant tumours (growth rate 147.6 mIU/mg/day vs. 38.7 mIU/mg/day over one week period; $p<0.001$; mean doubling time 3 vs. 7 days, respectively). Tumour/liver mass was significantly higher in Late Resistant vs. Sensitive tumours ($p<0.01$) in agreement with secreted hCG levels (ANOVA $p<0.0001$, data not shown).

Control tumours typically grew as single red, smooth, hemorrhagic nodules and sorafenib-treated tumours became white and irregular-shaped (Figure 3.1B). Sorafenib-treated tumours were highly necrotic except for the tumour rim adjacent to the liver or small viable islands or tracks of tumour cells throughout the core. Control and Stop tumours were mostly viable and filled with red blood cell pools (Figure 3.1C). Overall sorafenib treatment significantly increased the %necrotic tumour tissue (Control vs. Early resistance $p<0.01$ or vs. Late Resistance $p<0.001$; Figure 3.1C). Initial treatment did not significantly reduce tumour cell proliferation (Control vs. Sensitive $p>0.05$, ANOVA $p=0.01$) or induce apoptosis in tumour cells (ANOVA $p=0.80$; Figure 3.1D-E). Resistance was not associated with significant induction of proliferation nor reduced apoptosis (Figure 3.1D-E) and ERK phosphorylation remained unchanged (Figure 3.1F), altogether highlighting minimal direct tumour targeting by sorafenib.

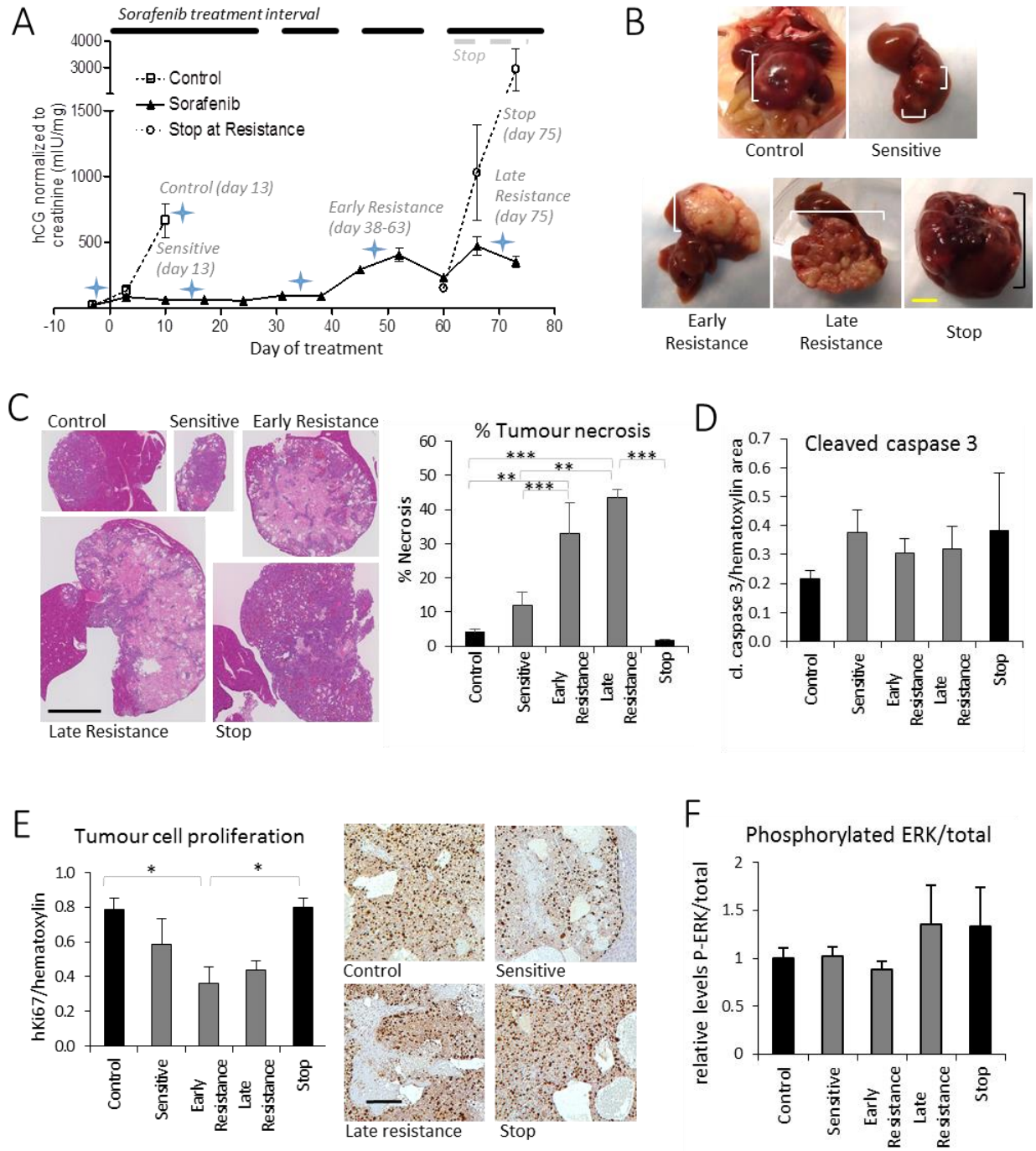



**Figure 3.1** Characteristics of sorafenib-resistant HCC xenografts. **A**. Tumour growth (based on urinary hCG/creatinine measurement) from mice bearing orthotopic Hep3B-hCG tumours while treated with vehicle or 30 mg/kg/day oral sorafenib. The treatment schedule is indicated. Mice were imaged (star symbol) or sacrificed at the indicated time points to study sensitive and resistance disease. Therapy was stopped for two weeks in a subgroup of mice after the development of resistance to study resistance reversibility. **B**. Hep3B-hCG tumours (square brackets)

are shown in situ and excised with liver intact. Note that sorafenib treated tumours are non-hemorrhagic and irregular. Scale bar=5 mm. **C**. Representative H&E stained tumours are shown from each of the tumour groups analyzed. The percent of tumour necrosis significantly increased over time during treatment unless therapy was discontinued (ANOVA $p<0.0001$). Scale bar=5 mm. **D**. No significant changes in tumour cell apoptosis were observed from immunohistochemical staining for cleaved caspase 3 (ANOVA $p=0.80$). **E**. Tumour cells regardless of treatment had a high proliferative index based on human Ki67 immunostaining (images at left) with a trend toward decreased cell proliferation during initial sorafenib treatment (Sensitive; ANOVA $p=0.01$). **F**. Levels of phosphorylated ERK1/2 (Thy202/Tyr204) normalized to total ERK1/2 did not significantly change during sorafenib treatment (ANOVA $p=0.62$). Scale bars= 100 µm. N=6/group. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

## Sorafenib treatment alters the microcirculation of orthotopically implanted HCC tumours

We examined whether re-induction of angiogenesis[4,5] in the tumour microenvironment might explain acquired resistance to sorafenib therapy. Tumours were dual stained for CD34, a marker of immature HCC neovessels but not sinusoidal vessels[23,24], and the pericyte marker α-smooth muscle actin (αSMA). CD34+ microvessels were abundant in Control tumours but were significantly depleted in responding Sensitive tumours and remained so during Early and Late Resistance (Figure 3.2A) unless therapy was stopped. Resistant tumours lacked CD34+ vessel hotspots. CD34 vessel immunoreactivity was generally low or absent in Resistant tumour vascular structures; rather, such vessels were frequently associated with auto-fluorescent cells reminiscent of hepatocytes (Figure 3.2A-B). Vessel quantification indicated a significant and maintained depletion of CD34+ microvessels during sorafenib treatment (ANOVA $p<0.0001$, $p<0.001$ for sorafenib Sensitive and Resistant relative to Control or Stop groups). Stopping therapy caused CD34+ vessels to return to Control levels (Figure 3.2C). The proportion of CD31 and Ki67-positive 'proliferating vessels' was also significantly reduced by sorafenib (ANOVA $p<0.0001$, $p<0.001$ Control vs. Sensitive, or $p<0.0001$ vs. Late Resistance) unless therapy was stopped (Figure 3.2D).

We observed a minor change in pericyte-coated vessels across groups (positive for both αSMA and CD34; ANOVA p=0.03) but the difference was not significant between Sensitive and Early Resistance time points (p=0.17) therefore enhanced vessel maturation[4,5] did not appear to explain the onset of resistance (Figure 3.2E). However unlike other groups, Stop tumour CD34+ vessels were frequently surrounded by thick layers of αSMA+ myofibroblasts (NG2-negative; NG2+ pericytes also surrounded these vessels, data not shown) (Figure 3.2F). These myofibroblasts were abundant in Stop tumours and formed extensive networks rich in CD34+ microvessels. Sparser myofibroblasts were found to a much weaker extent in Late Resistant tumours.

As a result of angiogenic inhibition, sorafenib treatment led to a widespread increase in expression of the hypoxia marker carbonic anhydrase IX (CAIX; 4.5-fold in Sensitive vs. Control $p<0.01$; ANOVA p=0.002; Figure 3.2G-H). CAIX expression appeared most extensive in Sensitive tumours suggesting partial improvement of tumour tissue oxygenation during resistance, but such changes were not statistically significant ($p>0.05$). CD34+ vessels were rarely localized central to CAIX+ tumour cells in Resistant tumours.

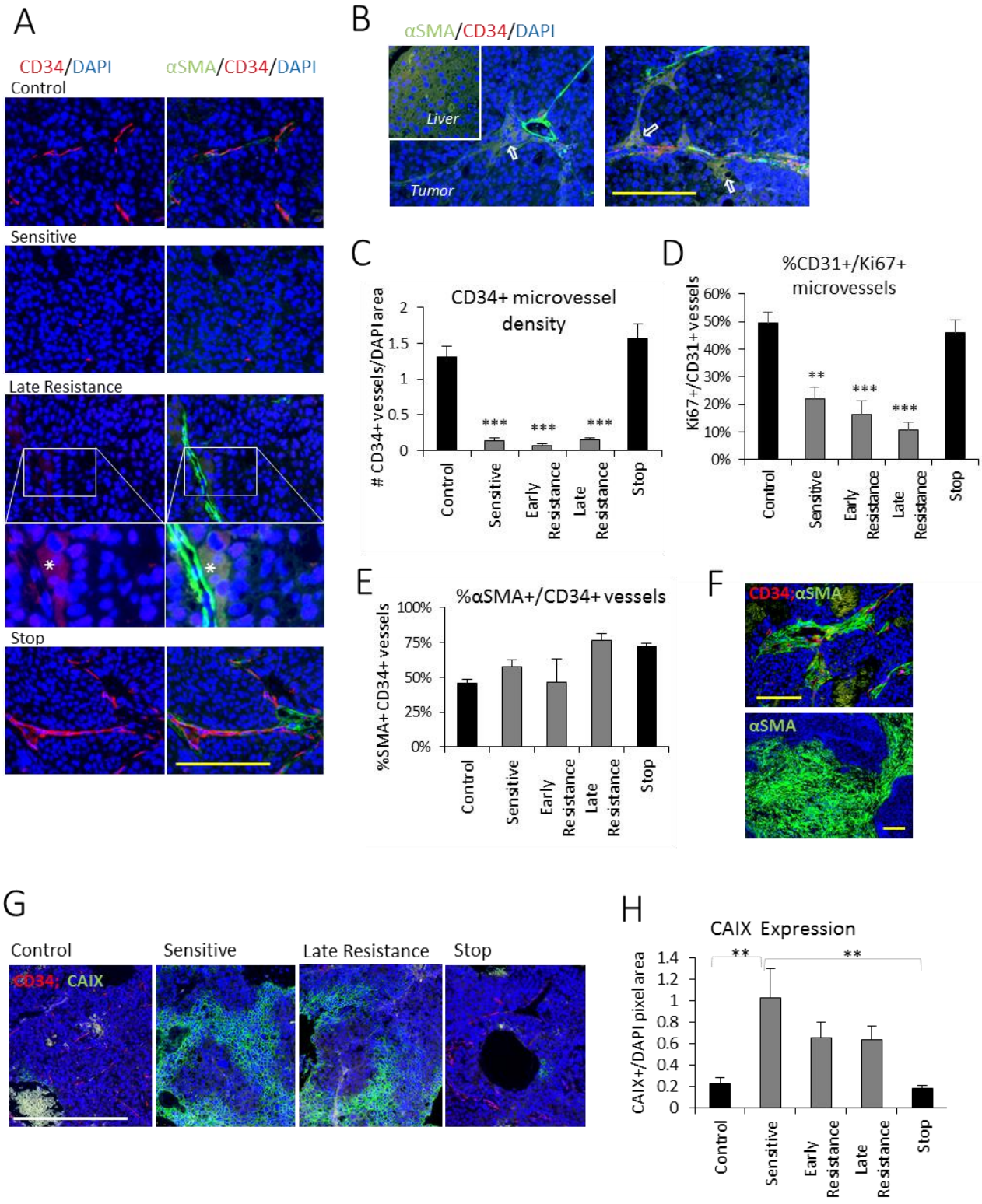

A
CD34/DAPI
αSMA/CD34/DAPI
Control
Sensitive
Late Resistance
Stop
B
αSMA/CD34/DAPI
Liver
Tumor
C
CD34+ microvessel density
# CD34+ vessels/DAPI area
Control
Sensitive
Early Resistance
Late Resistance
Stop
D
%CD31+/Ki67+ microvessels
Ki67+/CD31+ vessels
E
%αSMA+/CD34+ vessels
%SMA+CD34+ vessels
F
CD34;αSMA
αSMA
G
Control
Sensitive
Late Resistance
Stop
CD34; CAIX
H
CAIX Expression
CAIX+/DAPI pixel area

**Figure 3.2** Tumour angiogenesis during sorafenib treatment. **A**. Immunofluorescent images of Hep3B-hCG angiogenic microvessel density by tumour staining for CD34+ microvessels (red), αSMA pericytes (green) and nuclei (DAPI; blue). CD34/DAPI merge are shown in the left column and CD34/αSMA/DAPI merge at right. Note abundant CD34+ microvessels only in Control and Stop tumours. During resistance CD34-/αSMA+ vessels adjacent to autofluorescent cells become evident (*), shown in the inset. **B**. Such vessels were often surrounded by large regions of these autofluorescent cells which appear to be hepatocytes (liver shown in inset). Late Resistant tumours are shown. **C**. CD34+ microvessel density normalized to DAPI significantly decreased during treatment and rebounded after stopping therapy (ANOVA $p<0.0001$, ***$p<0.001$ vs. control or stop groups). **D**. The proportion of tumour microvessels (CD31, green) containing proliferative endothelial cells (Ki67+) was also suppressed throughout treatment (ANOVA $p<0.0001$, **$p<0.01$ and ***$p<0.001$ vs. control or stop groups). **E**. The %αSMA+ covered vessels increased marginally from Sensitive to Late Resistance time points (T-test $p=0.02$) but not from Sensitive to Early Resistance ($p=0.17$; ANOVA $p=0.03$, $p>0.05$ for multiple comparisons). **F**. Abundant αSMA+ myofibroblasts (green) were observed in Stop group tumours and contained a high density of CD34+ microvessels (red; top). **G**. Immunofluorescence for CAIX (green) demonstrated marked increases in CAIX-positive area in sorafenib-treated tumours, H. which was significantly upregulated during treatment ANOVA $p=0.002$; **$p<0.01$. Scale bars: 200 μm (yellow) and 500 μm (white). N=6/group. Blue: DAPI.

## Ultrasound imaging reveals no evidence for rebound perfusion during resistance

We used ultrasound imaging to examine changes in tumour perfusion. The microbubble contrast agent used (1-10 μm diameter) remains strictly intravascular, allowing accurate assessment of tumour perfusion without the confounding variable of vessel permeability as detected by CT or MRI[25]. Analogous to CD34+ microvessel densities, pre-treatment and control Hep3B-hCG tumours were highly perfused (Fig 3.3A) and contrast uptake was dramatically reduced following two weeks of treatment and remained so for subsequent weeks (Figure 3.3B).

To quantify global perfusion changes, peak enhancement (PE; the difference between maximum amplitude and the baseline) and wash-in rate (WIR; the maximum slope of the curve) were taken from the time-intensity curve. These are indicators of tumour blood volume and blood flow rate, respectively. PE and WIR each declined significantly (62% and 60% decrease, respectively) after initial sorafenib

treatment but remained relatively unchanged afterwards (pre-treatment vs. week 2 $p<0.001$, Repeated measures one-way ANOVA $p<0.0001$; Figure 3.3C-D), indicating no evidence of a rebound re-perfusion with sorafenib resistance.

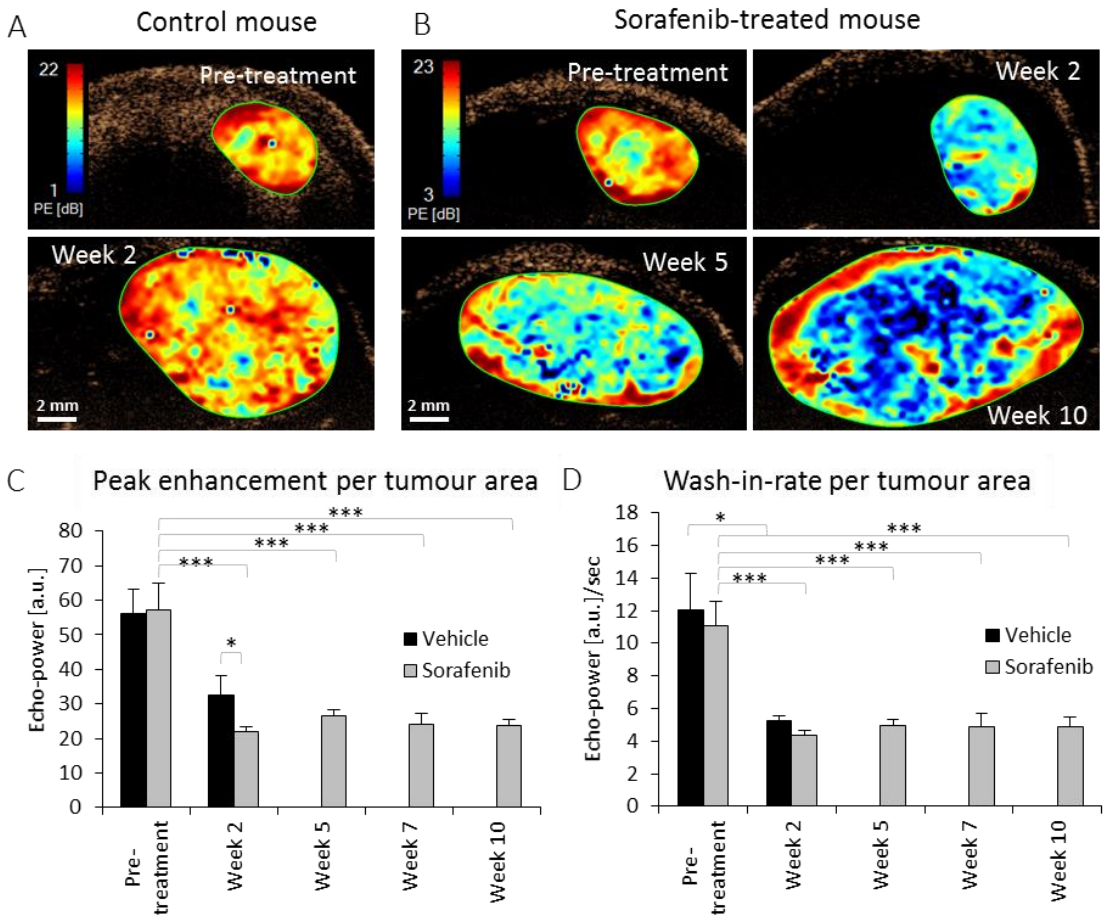
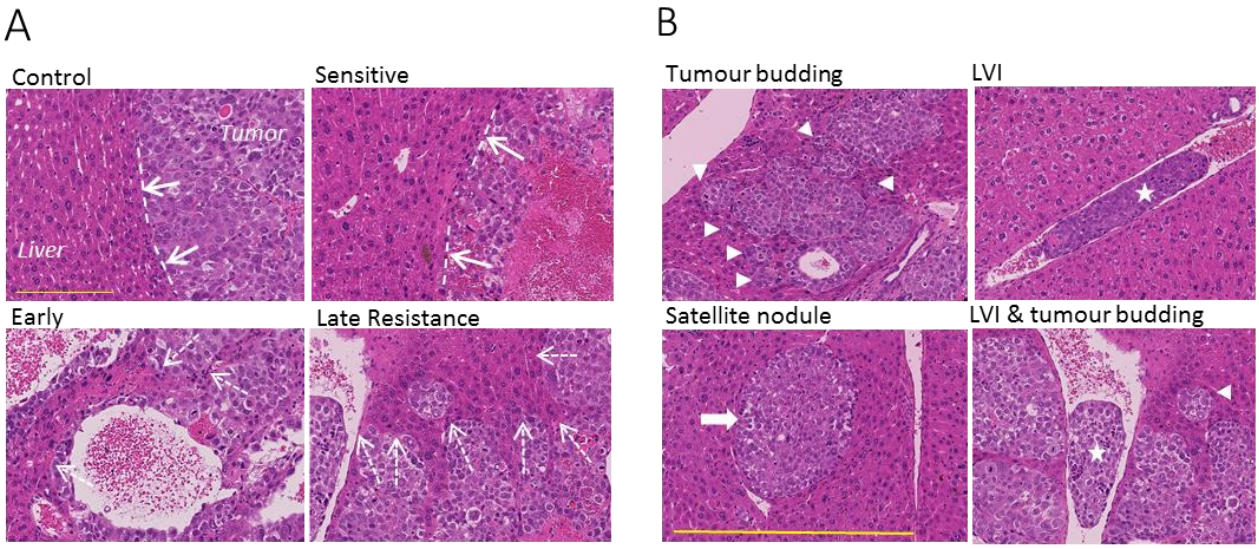



**Figure 3.3** Conventional analysis of HCC tumour perfusion during sorafenib treatment using contrast-enhanced ultrasound imaging. Representative parametric maps of peak enhancement (PE), an indicator of tissue blood volume, shows changes in tumour perfusion in a **A**. vehicle treated and **B**. Sorafenib treated mouse. Red represents regions of high PE, blue are areas of low PE, and black represents no perfusion. **C**. PE averaged for each tumour decreased significantly 2 weeks after Sorafenib therapy (Repeated measures one-way ANOVA $p<0.0001$, ***$p<0.001$ vs. Pre-treatment), but further changes were insignificant for the following weeks. No significant changes occurred with vehicle treated mice (Paired t-test $p=0.07$), whereas a significant change was observed between vehicle and Sorafenib treated mice in week 2 (Unpaired t-test *$p<0.05$). Vehicle control mice were sacrificed after week 2. **D**. Wash-in rate used as an indicator of rate of blood flow showed a lack of significant changes throughout treatment

weeks, including periods of drug resistance. Significant changes were only observed between pre- and post-treatment for both Sorafenib (Repeated measures one-way ANOVA $p<0.0001$, ***$p<0.001$) and vehicle (Paired t-test *$p<0.05$) treated groups. N=4 (vehicle), 11 (sorafenib).

## Histopathological evidence for increased invasiveness in sorafenib-treated HCC tumours

Based on histopathological criteria from liver metastases and HCCs[10,26], we observed that Control and Sensitive tumours predominantly had a 'pushing' growth pattern characterized by compression of the hepatocyte plates parallel to the tumour-liver interface (Figure 3.4A). By Early Resistance the tumour growth pattern became highly infiltrative. Tumour 'buds' (cell clusters of ≤5 cells in diameter[27]) and tumour nests formed along the invasive front resulting in the incorporation of liver parenchyma into the tumour (Figure 3.4B). We also observed a trend of increased lymphovascular invasion and hepatic satellite nodule development during resistance (plus one case of distant abdominal metastases; Figure 3.4B-C), additional signs that resistant tumour cells became highly invasive.




C

| Group | Pushing growth pattern | Infiltrative growth pattern | Satellite nodules | Mean # satellite nodules | Lymphovascular invasion | Tumour budding | Mean # tumour buds |
|---|---|---|---|---|---|---|---|
| Control | 6/6 | 0/6 | 2/5 | 0.6 | 0/5 | 4/6 | 1.8 |
| Sensitive | 5/6 | 1/6 | 0/5 | 0 | 0/5 | 5/6 | 2.7 |
| Early Resistance | 0/6 | 6/6 | 4/6 | 1.5 | 2/6 | 6/6 | 4.7 |
| Late Resistance | 0/6 | 6/6 | 5/5 | 6.6 | 3/5 | 6/6 | 9.7 |
| Stop | 3/6 | 3/6 | 3/5 | 4.6 | 5/5 | 5/5 | 12.8 |

**Figure 3.4** Histopathological signs of invasion in HCC xenografts. **A**. Tumour growth patterns switched from predominantly pushing (Control and Sensitive tumours, dashed lines and solid arrows) to infiltrative (Resistance phases) leading to tumour incorporation of liver parenchyma (dashed arrows). The inset (top left) shows hepatocytes invaginated by a control tumour, an effect that is amplified during resistance. **B**. Tumour budding (triangle), lymphovascular invasion (LVI; star) and satellite nodules (arrow), additional signs of tumour aggressiveness, were common in resistant tumours. **C**. Some of the invasive tumour features tended to increase from Control and Sensitive to Resistant tumours, with a mixed phenotype in Stop group tumours. N=6/group. Scale bar=200 µm.

## Resistance to sorafenib is associated with increased dependency on vessel co-option

We hypothesized that invasive tumour growth and incorporation of liver parenchyma in the absence of angiogenesis re-induction meant that sorafenib-treated HCC tumour cells were co-opting the

normal liver vasculature[13]. Tumours were co-stained for CD31 (to detect all blood vessel types), human lamin A/C (which detects the human cancer cells) and then counterstained lightly with hematoxylin and eosin to reveal normal anatomical liver structures (Figure 3.5A). This allowed us to identify five distinct types of vessels in Hep3B-hCG tumours: (1) tumour-embedded vessels (TV), which were defined as CD31+ vessels bordered only by lamin A/C+ tumour cells (Figure 3.5B); (2) connective tissue vessels (CTV) which were CD31+ vessels bordered by fibroblasts (Figure 3.5B); (3) hepatocyte vessels (HV), which were CD31+ vessels bordered by hepatocytes (Figure 3.5C); (4) hepatic central veins (CV; Figure 3.5D); and (5) normal vessels of the portal triads (PT; Figure 3.5E). Since vessels types 3-5 are all normal vascular structures of the liver, their presence *within* the tumour mass is evidence for vessel co-option. CTVs and HVs appeared to correspond with many of the myofibroblast αSMA+ and CD34-negative vessels, respectively, observed in prior histological analyses (Figure 3.2A-B).

We examined the abundance of these vessel types in Control and sorafenib-treated tumours. TVs prevailed in Control tumours (58.6 ±11.5 vessels/mm$^2$ viable tumour area, T-test p=0.01 vs. HVs) but were drastically reduced to 7.3 ±1.8, 3.2 ±0.8 and 3.8 ±0.7 vessels/mm$^2$ ($p<0.001$) during Sensitive, Early and Late Resistance phases, respectively. These vessels then rebounded to 46.4 ±4.1 vessels/mm$^2$ in Stop tumours (ANOVA $p<0.0001$ (Figure 3.5F). Similar data was obtained by normalization to total tumour area (data not shown). Following initial depletion by treatment, CTVs rebounded dramatically after resistance when therapy was stopped (3.9 ±0.8 during Late Resistance to 22.2 ±6.2 vessels/mm$^2$ ANOVA $p=0.0002$, $p<0.001$).

In contrast, the quantity of HVs remaining relatively stable across groups (ranging from 13.6 (Sensitive) to 22.8 (Stop) vessels/mm$^2$, ANOVA p=0.53), indicating their relative resistance to therapy. However during Early Resistance HVs significantly outnumbered TVs (T-test at Early Resistance p=0.001, p=0.01 at Late Resistance; Figure 3.5F). Hepatocytes in Control/Sensitive tumours tended to be highly mixed with tumour cells resulting in most HVs associated with scarce hepatocytes and in Resistant

tumours larger areas of liver parenchyma and sinusoids were characteristic of these vessels (Figure 3.5C). In addition, portal triads (PT) and central veins (CVs) were found in all tumours across all groups. Unlike HVs their levels were highly variable with a trend toward differences across groups (ANOVA $p=0.14$ and $p=0.08$, respectively; Figure 3.5G). Relative to Control tumours, the incidence of PT and CV increased significantly by Late Resistance (T-test, $p=0.003$ and $p=0.004$, respectively). HVs generally disappeared in the necrotic cores of Resistant tumours, but large liver vessels appeared more resilient and could be found throughout such tumours.

Importantly, HCC tumours switched to a blood supply provided by vessel co-option during sorafenib treatment, with 23.3 ±4.2% of total vessels provided by vessel co-option in Control tumours to as high as 75.0 ±4.4% of total vessels during Early Resistance ($p<0.001$) (Figure 3.5H). Stopping therapy caused a return of co-opted vessels to baseline (24.9 ±5.0%, ANOVA $p<0.0001$; Figure 3.5H).

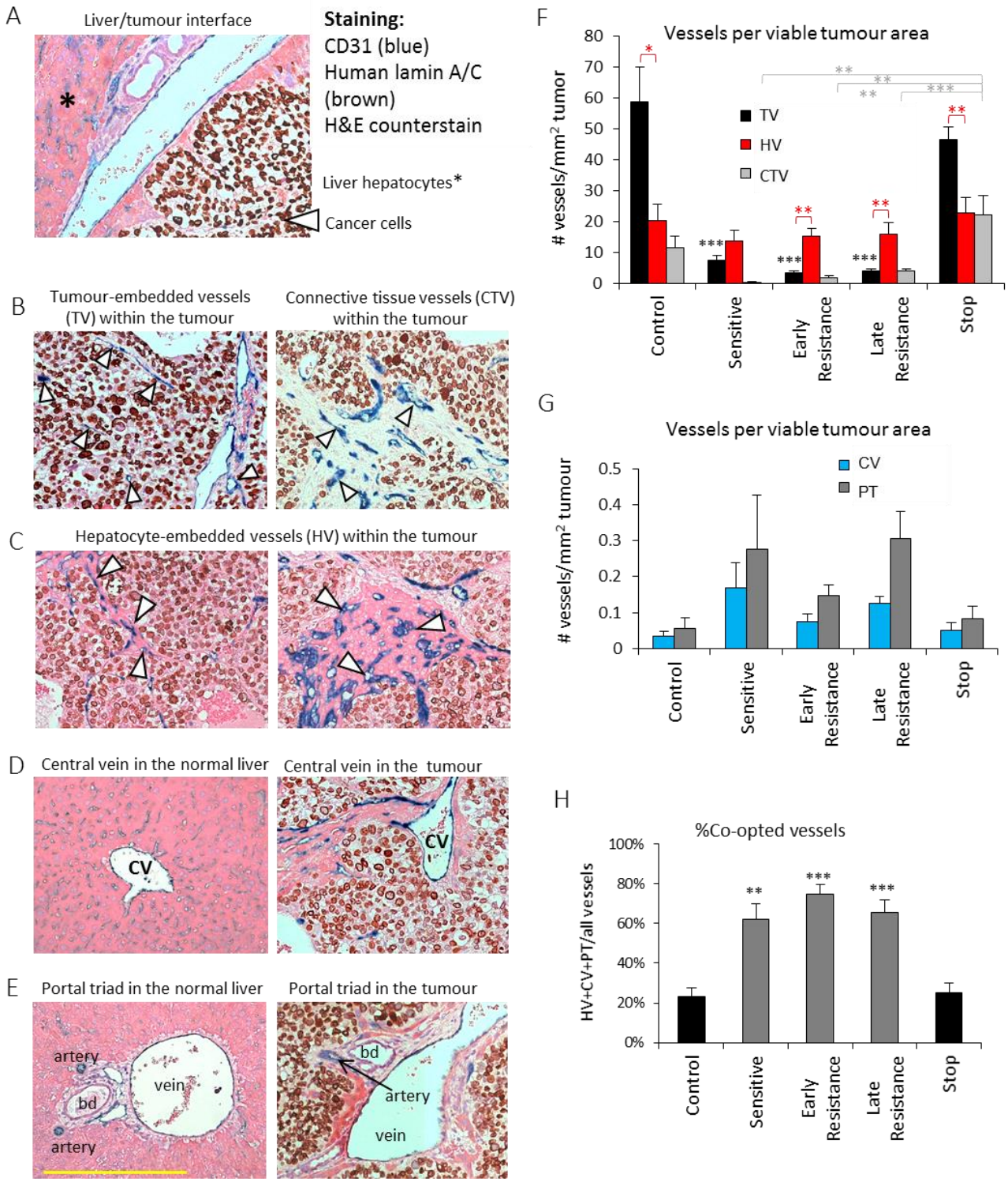

A
Liver/tumour interface
Staining:
CD31 (blue)
Human lamin A/C (brown)
H&E counterstain
Liver hepatocytes*
Cancer cells
B
Tumour-embedded vessels (TV) within the tumour
Connective tissue vessels (CTV) within the tumour
C
Hepatocyte-embedded vessels (HV) within the tumour
D
Central vein in the normal liver
Central vein in the tumour
CV
E
Portal triad in the normal liver
Portal triad in the tumour
artery
vein
bd
F
Vessels per viable tumour area
# vessels/mm² tumor
TV
HV
CTV
Control
Sensitive
Early Resistance
Late Resistance
Stop
G
Vessels per viable tumour area
# vessels/mm² tumour
CV
PT
H
%Co-opted vessels
HV+CV+PT/all vessels

**Figure 3.5** Evidence of liver vessel co-option in HCC xenografts. **A**. Tumour sections were stained for human lamin A/C (brown), H&E and CD31 (blue) to differentiate between co-opted liver parenchyma- and tumour-derived vessels. **B**. Non-co-opted vessels, the tumour-embedded vessels (TV) and connective tissue vessels (CTV) are surrounded by tumour cells or fibroblasts, respectively. **C**. Hepatocyte-embedded vessels (HV) were the most common microvessel structures found in Hep3B-hCG tumours, varying from single-layered hepatocytes (left) or large patches of hepatocytes associated with CD31+ vessels (right). **D**. Central veins (CV), shown in the liver (left) were also observed in the tumour. **E**. The vessels of portal triads (PT) were also observed in the liver (left) and portal triads (PT) as well as in the tumour. **F**. TVs were characteristic of Control tumours and they diminished during sorafenib treatment (ANOVA $p<0.0001$, ***$p<0.001$ vs. Control and Stop groups) after which HVs predominated (ANOVA $p=0.53$). T-test results within groups are shown in red for HV vs. TV comparisons. After initial inhibition, CTV tended to re-emerge over time and after stopping therapy (ANOVA $p=0.0002$). **G**. Tumour-incorporated PT and CV tended to increase in prevalence during treatment (ANOVA $p=0.14$ and $p=0.08$, respectively). **H**. The % of co-opted (HV+CV+PT) out of total vessels significantly increased during sorafenib treatment. bd=bile duct. Scale bar 300 µm; n=5-6/group. Triangle = vessel type indicated. *$p<0.05$, **$p<0.01$, ***$p<0.0001$.

## Contrast-enhanced ultrasound imaging of vessel co-option

We evaluated whether higher resolution contrast-enhanced imaging might differentiate between tumour perfusion by angiogenesis vs. vessel co-option. Out-of-plane frames were removed from contrast enhancement cineloops and perfusion parameters were extracted from 3x3 pixel areas, a methodology that improved image resolution. Networks of large vessels were thus exposed in sorafenib-treated tumours spanning the tumour core. Structurally similar networks were visible within the same mice across time points, suggesting that some of these vessels persisted from prior to sorafenib treatment (Figure 3.6B). A highly perfused (high-contrast) thin tumour rim developed after 5 weeks of sorafenib treatment.

Based on histological sections of a parallel subset of tumours, the uniform high-contrast enhancement regions of control tumours corresponded to areas primarily occupied by tumour-embedded vessels (TVs), whereas the hyper-intense rim of resistant tumours mapped to the hepatocyte vessel (HV)-

dense invasive tumour front, which rarely contained TVs (Figure 3.6C). Large vascular structures and non-perfused (black) tumour regions corresponded to large liver vessels and necrotic areas, respectively. Thus, the functional vasculature (based on PE maps) correlated well with histological features including the abundance of co-opted vs. non-co-opted vessel subtypes (Figure 3.5F-H). Notably, high tumour rim enhancement did not reflect regions of angiogenic rebound, but rather areas of active vessel co-option.

A wide range of peak enhancement values were observed in both the tumour and the liver, with distinct differences between the overall distributions (Figure 3.6D). Following initial sorafenib treatment, PE distributions shifted dramatically relative to untreated tumours (Figure 3.6D): a significant increase in the frequency of the lowest PE intervals (e.g. to smaller capillaries, poorly or non-perfused/necrotic tissues) as well as a decrease in 27 of the other 29 PE intervals ($p<0.01$) was observed. Over weeks 2-10 of prolonged sorafenib therapy, PE distribution remained the same (Figure 3.6E). Distribution of functional vessels did not take on Control-like patterns, further supporting a lack of rebound angiogenesis during treatment, whereas the absence of change in the PE distribution in the lowest intervals mimicked the stability of HV densities. Thus analysis of measured relative blood volume (PE) using contrast ultrasound shows patterns consistent with vessel co-option but not angiogenesis.

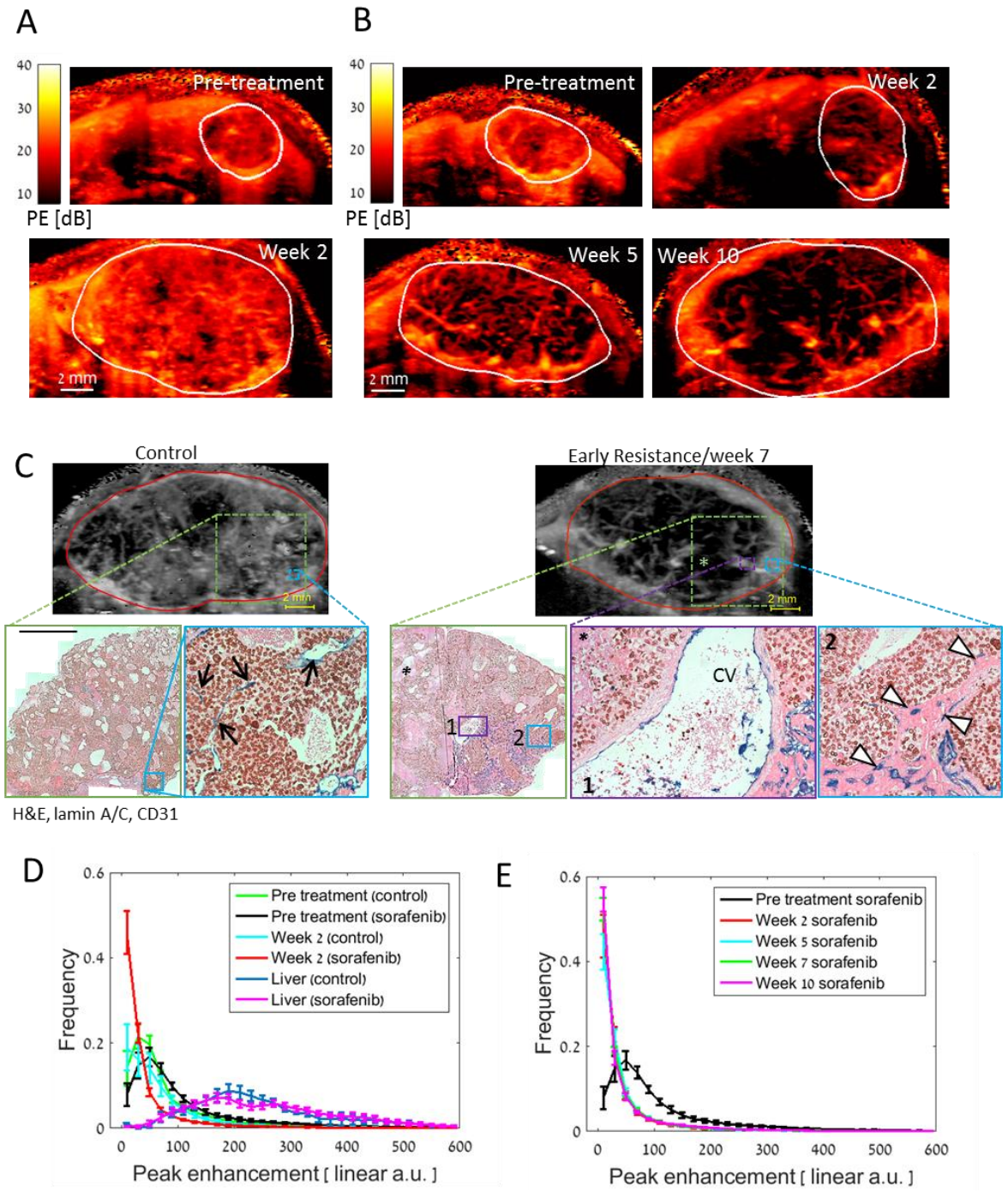



**Figure 3.6** Local perfusion analysis of sorafenib-treated HCC tumours. **A**. Representative peak enhancement (PE) maps of a vehicle control-treated mouse pre-treatment and after 2 weeks. **B**. PE maps of a sorafenib-treated mouse weeks 2-10. Resistance typically occurs weeks 5-6. **C**. Examples of contrast-enhanced ultrasound images (top) and corresponding histological sections and features of HCC tumours stained for CD31 (blue), human lamin A/C (brown) and H&E. The expanded regions (boxes) demonstrate that high tumour-embedded vessel (TV, black arrows) or hepatocyte vessel (HV, white triangles) densities correspond to high-contrast regions in control vs.

sorafenib-resistant tumours, respectively (scale bar=2 mm). Large vessels evident in sonograms resemble the large liver vessels within the tumour, such as central veins (CV), shown here. *=necrotic region. **D**. Histograms of the local PE values in control and sorafenib-treated tumours are shown pre-treatment and after 2 weeks of therapy. Following treatment, the frequency of the PE measurements in the lowest interval increased while the values in 27 of the other 29 PE intervals reduced significantly ($p<0.05$). Changes in the PE value distribution of the control group were not significant ($p>0.05$). PE distribution in the normal liver is indicated by the dashed lines. **E**. Similar distributions of PE values were observed between weeks 2, 5, 7 and 10 on sorafenib therapy. The mean values ± SEM are presented for every interval of PE values.

## Tumour biomarkers of sorafenib resistance

Motivated by the lack of clear associations of protein biomarkers and patient response/resistance to anti-VEGF therapy[5], we evaluated expression of several growth factors and cytokines in Hep3B-hCG tumour lysates. Surprisingly, levels of four were significantly associated with treatment (human VEGF-A) or treatment resistance (mouse VEGF-A, mouse osteopontin (OPN) and mouse Ang2), despite near-complete inhibition of angiogenesis during these time points (Table 1). Mouse VEGF-A, mouse OPN and human VEGF-A each returned to baseline after stopping treatment. Interestingly, each of these three factors correlated negatively with tumour-embedded vessel (TV) density and positively with percentage of vessel co-option, but the opposite was observed for mouse Ang2 (Figure 3.7).

**Table 3.1** Tumour concentrations of angiogenesis-associated growth factors and cytokines. Concentrations normalized to total tumour protein +/- standard error with ANOVA p value are indicated. Values significantly different ($p<0.05$) from Control and Sensitive tumours are indicated by an asterix (*) or underlined, respectively. Significant changes in three host-derived factors and one tumour-cell derived pro-angiogenic factor were observed. Tumour cell bFGF, factors involved in vessel integrity (mouse Ang1, PDGF-AB, PDGF-BB) or bone marrow cell-mobilizing cytokines did not significantly change (mouse G-CSF and SDF-1; ANOVA $p>0.05$). N=6/group.

| Factor | Group | | | | | Units | P Value |
|---|---|---|---|---|---|---|---|
| | **Control** | **Sensitive** | **Early Resistance** | **Late Resistance** | **Stop** | | |
| human bFGF | 85.7 ± 10.2 | 101.3 ± 19.5 | 132.8 ± 14.5 | 127.9 ± 6.1 | 114.7 ± 20.2 | pg/mg | 0.23 |
| **human VEGF-A** | 95.9 ± 8.4 | **973.7 ± 131.8*** | **938.8 ± 111.7*** | **1,396.9 ± 252.3*** | 106.2 ± 22.3 | ng/mg | **<0.0001** |
| **mouse VEGF-A** | 32.3 ± 8.4 | 139.8 ± 30.3 | 390.5 ± 60.2 | **696.1 ± 125.4*** | 137.7 ± 31.6 | ng/mg | **<0.0001** |
| mouse Ang1 | 75.0 ± 16.0 | 146.1 ± 25.4 | 99.1 ± 34.8 | 73.6 ± 15.0 | 96.8 ± 33.4 | pg/mg | 0.28 |
| **mouse Ang2** | 207.1 ± 29.9 | 50.1 ± 10.4 | 131.5 ± 35 | 231.7 ± 70.3 | **538.7 ± 134.3*** | pg/mg | **0.0007** |
| mouse G-CSF | 129.2 ± 18.4 | 275.8 ± 83.1 | 836.2 ± 407.2 | 128.6 ± 22.4 | 524.7 ± 343 | pg/mg | 0.21 |
| **mouse OPN** | 1.6 ± 0.4 | 2.4 ± 0.2 | 7.0 ± 0.8 | **15.5 ± 3.2*** | 4.1 ± 0.9 | ng/mg | **<0.0001** |
| mouse SDF-1 | 82.5 ± 10.3 | 112.9 ± 11.5 | 90.1 ± 13 | 119 ± 12.1 | 104.5 ± 16.1 | pg/mg | 0.25 |
| mouse PDGF-AB | 6.9 ± 0.7 | 6.3 ± 0.8 | 6.8 ± 1.5 | 6.0 ± 0.5 | 6.3 ± 0.9 | pg/mg | 0.96 |
| mouse PDGF-BB | 14.5 ± 2.2 | 27.5 ± 5.6 | 38.4 ± 11.5 | 22.9 ± 3.1 | 14.2 ± 2.5 | pg/mg | 0.05 |

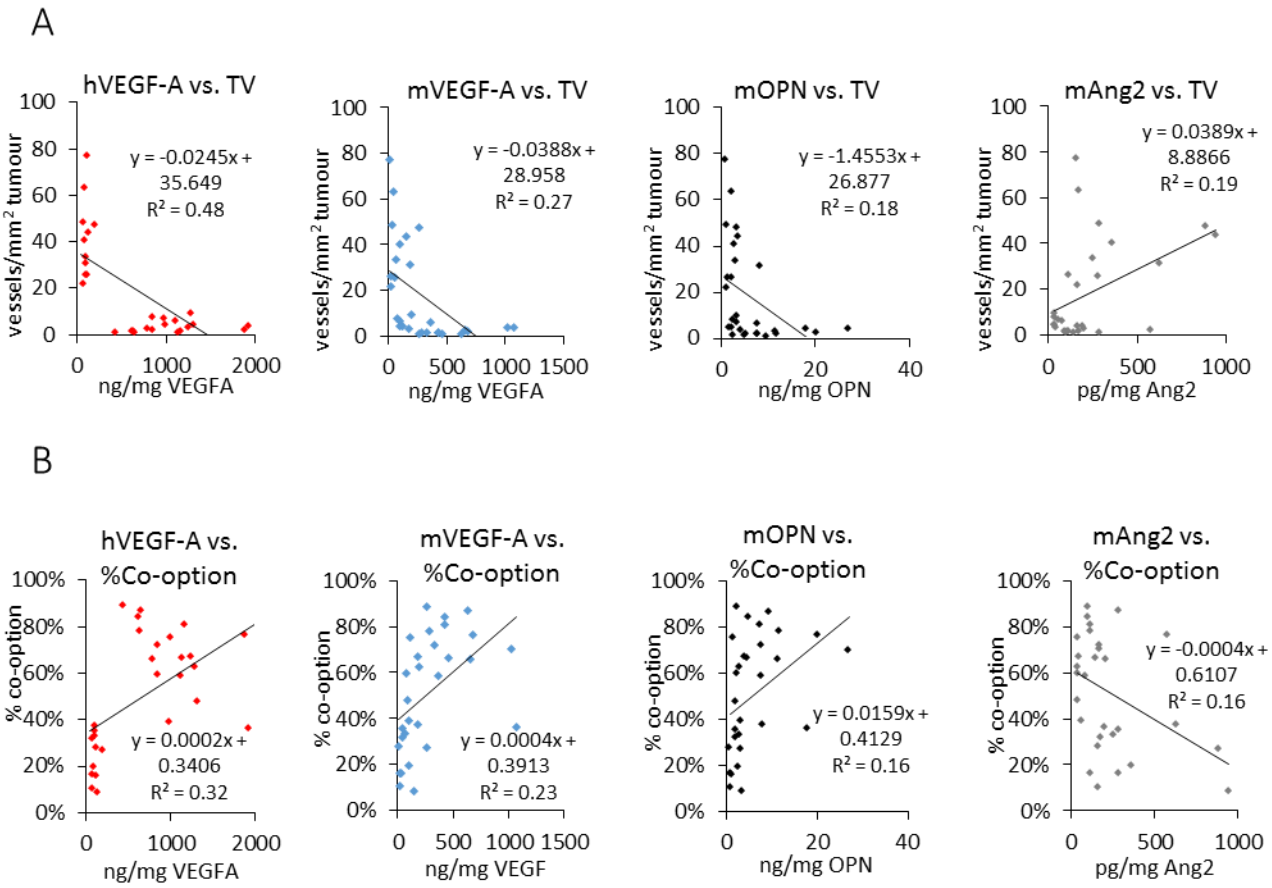


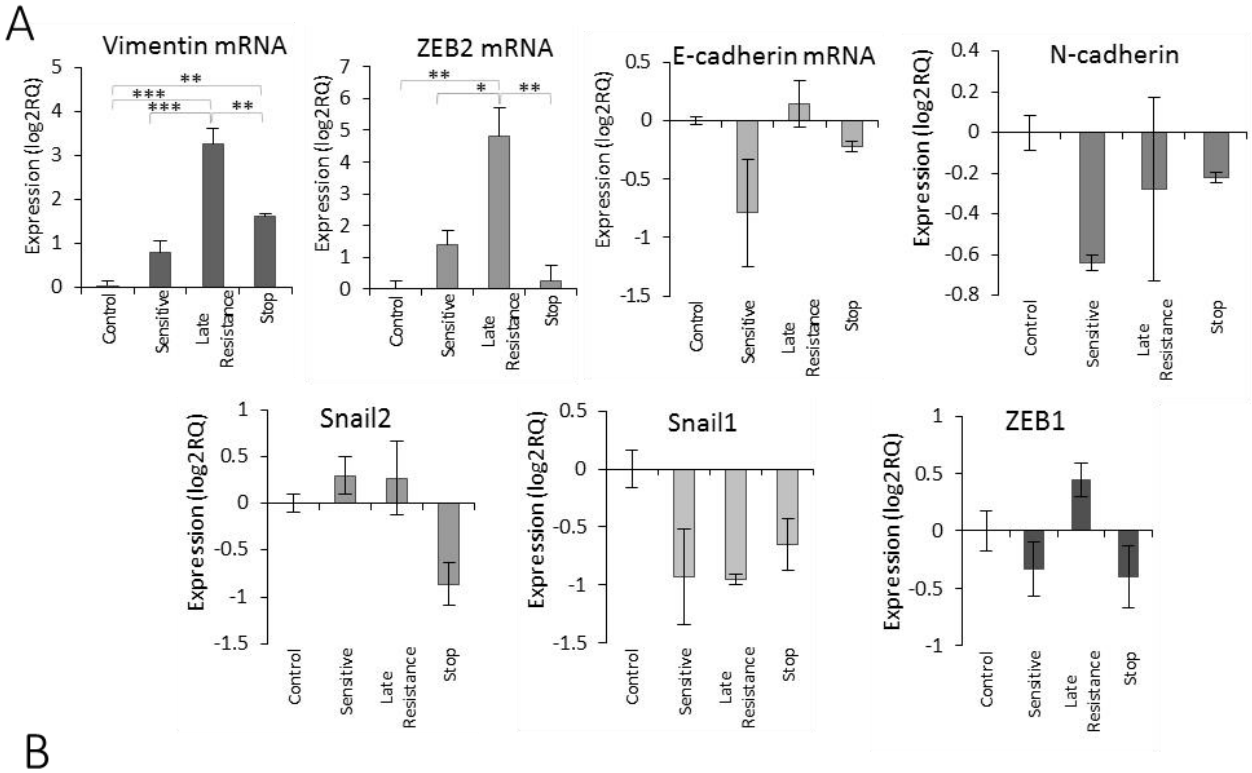

**Figure 3.7** Correlation of pro-angiogenic growth factors with angiogenesis and vessel co-option. Levels of significantly changing pro-angiogenic factors in tumor lysates were correlated with tumor vascularity parameters as an exploratory analysis. **A**. TV (tumour-embedded vessel) counts and **B**. percent of co-opted vessels (hepatocyte vessels, portal triads and central veins) out of total vessels were each correlated to tumour expression of significantly changing growth factors. Only statistically significant correlations are shown ($p<0.05$). TV and %co-option values were normalized to total tumour area. The Pearson R correlation coefficient and the linear regression equation are indicated. The strongest negative correlation was between human (h)VEGF-A levels and TV ($R^2=0.48$). The strongest positive correlation was between hVEGF-A levels and %co-option ($R^2=0.32$). N=6/group.

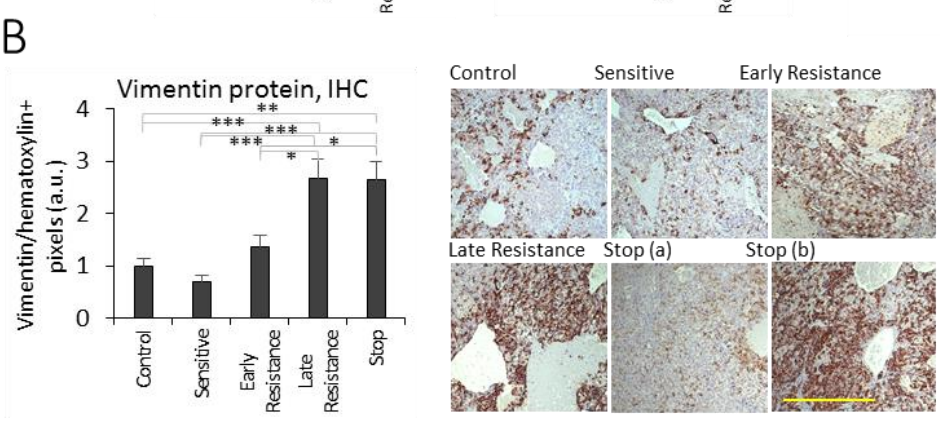
**EMT-like pathways are activated during resistance to sorafenib**

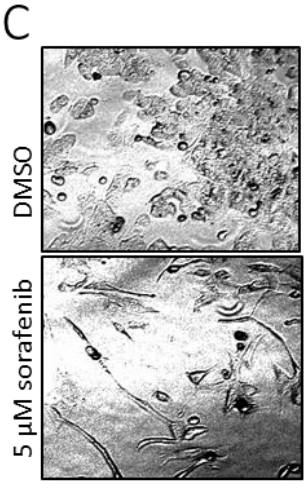
To elucidate potential molecular pathways underlying vessel co-option, we performed human microRNA sequencing on Control, Sensitive and Late Resistant tumours (n=2/group) and bioinformatic analysis to identify pathways that were upregulated by sorafenib treatment. Interestingly, 4 of the top 8 upregulated pathways were involved in cellular motility and invasion processes: axonal guidance, EMT, STAT3 and Wnt/β-catenin signaling (full analysis to be published elsewhere, Butz H et al.).

We further investigated EMT due to its reported involvement in anti-VEGF therapy resistance[5,28] and potential contribution to increased HCC invasion. STAT3 and β-catenin are also important transcriptional regulators and initiators of EMT in cancers including HCC[29-32]. Thus EMT may be a central mechanism behind sorafenib resistance. We validated expression of several EMT genes by RT-qPCR. In Late Resistant vs. Control tumours, we observed significant up-regulation of vimentin mRNA (a mesenchymal intermediate filament) and ZEB2 (a pro-EMT transcription factor; fold-increase of 3.26 ±0.36, $p<0.001$ for vimentin ANOVA $p<0.0001$, and 4.81 ±0.90, $p<0.01$ for ZEB2, ANOVA $p=0.001$) (Figure 3.8A). No significant changes in ZEB1, Snail1, E- or N-cadherin expression were observed (ANOVA $p>0.05$) with modest changes in Snail2 (ANOVA $p=0.04$; Figure 3.8A). Thus, the characteristic feature of EMT, the E to N 'cadherin switch' did not occur in tumour cells[29]. By immunohistochemistry we noted diffuse human vimentin protein expression in Control and Sensitive tumours which was expressed by the majority of cancer cells by Late Resistance (ANOVA $p<0.0001$, 3.8-fold increase $p<0.001$ Late Resistance vs. Sensitive; Figure 3.8B). Stop tumours were a hybrid of high and low-expressing areas, indicating partial reversal in vimentin expression upon discontinuation of sorafenib. Hep3B-hCG cells could adopt a mesenchymal spindle-shaped morphology during sorafenib treatment *in vitro* (Figure 3.8C), but despite expression of some EMT markers, they did not acquire this phenotype *in vivo* (Figure 3.4).

We tested whether chronic sorafenib treatment of an *in vivo*-derived resistant variant could transfer the resistance phenotype to new hosts, which was lot by drug withdrawal *in vitro* in previous studies[20]. This would implicate a link between treatment-induced maintenance of cancer cell invasion and resistance. Strikingly, chronically sorafenib-exposed resistant cells that were re-implanted intrahepatically into new hosts yielded a significantly greater incidence of large extra-hepatic metastasis compared to the same variant chronically treated with DMSO (Figure 3.8D, 62.5% vs. 0%, Fisher's exact test p=0.03). While this may in part be artefact since the rate of large metastasis is typically low in this model (~2%), chronic sorafenib exposure of cancer cells *in vitro* and *in vivo* did not lead to an earlier onset of acquired resistance (Figure 3.8E). Therefore other factors in the tumour microenvironment (e.g. the presence of liver parenchyma or hypoxia) must also contribute to resistance development. Taken together, sorafenib treatment led to molecular EMT-like changes following the drug-sensitive phase which coincided with invasive tumour growth and vessel co-option.



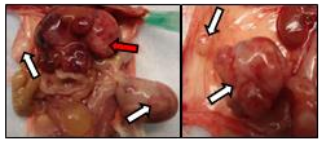




D

Extra-hepatic metastasis by resistant variant and mouse treatment

| In vivo / In vitro | Vehicle | Sorafenib |
|---|---|---|
| DMSO | 0 of 4 | 0 of 4 |
| SORAF | 3 of 4 | 2 of 4 |



SORAF to Vehicle

SORAF to Sorafenib

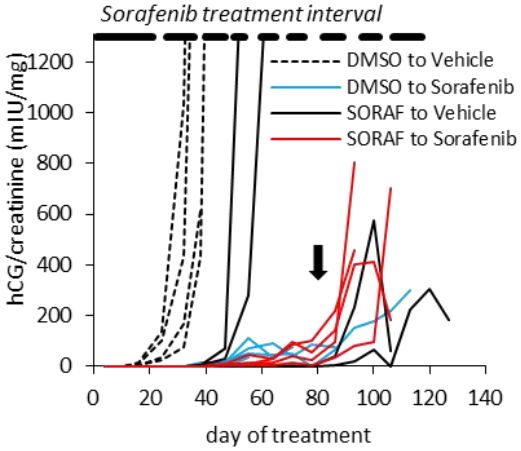


**Figure 3.8** Pro-invasive and EMT-like signaling in tumour cells during sorafenib resistance. **A**. Relative expression of EMT-associated genes indicated significant up-regulation of vimentin (ANOVA $p<0.0001$) and ZEB2 (ANOVA $p=0.001$) but no significant changes in E-cadherin (ANOVA $p=0.12$), ZEB1 (ANOVA $p= 0.08$), Snail1 (ANOVA $p=0.08$) or N-cadherin (ANOVA $p=0.34$). Snail2 levels significantly changed (ANOVA $p=0.04$), however group differences were not significant by multiple comparisons ($p>0.05$). N=3/group. **B**. Vimentin protein expression by immunohistochemical analysis was significantly up-regulated from Sensitive to Late Resistance phases, with a mixed phenotype in Stop tumours (ANOVA $p<0.0001$; scale bar: 500 µm; a.u.=arbitrary units). **C**. *In vitro* Hep3B-hCG parental cells took on a mesenchymal-like morphology during 48h 5 µM sorafenib treatment. **D**. Sorafenib exposure was maintained by chronically exposing *in vivo*-derived resistant tumour cells to 1.5 µM sorafenib (SORAF) or DMSO in culture prior to re-implantation into tumour-naïve hosts. *In vitro* sorafenib exposure significantly increased the incidence of metastasis in secondary hosts (Chi-Square test $p=0.03$). **E**. However, sorafenib re-treatment of hosts implanted with this chronically-treated variant did not accelerate the onset of acquired resistance. N=4/group. *$p<0.05$, **$p<0.01$, ***$p<0.0001$.

## DISCUSSION

Evidence from both patients and pre-clinical cancer models shows that many tumours can co-opt pre-existing blood vessels instead of utilizing angiogenesis[13]. However, the role of vessel co-option in acquired resistance to anti-angiogenic therapy has not been explored. Based on the evidence presented here, we propose that vessel co-option can facilitate reversible acquired resistance to anti-angiogenic therapy in HCC[20]. Upon sorafenib treatment, tumours are depleted of angiogenic vessels but co-opted pre-existing liver vessels in the tumour remain. The tumours become highly hypoxic and necrotic and tumour growth is stabilized. The cancer cells then undergo EMT-like molecular changes, which enables them to infiltrate the liver parenchyma, causing active recruitment and co-option of the liver vessels, ultimately facilitating resistance and resumption of tumour growth. Many of these changes are partially reversed by stopping therapy. Although it remains to be determined whether this mechanism occurs clinically, we have also observed vessel co-option as a mechanism of resistance to sorafenib in a second orthotopic HCC model using MHCC-97H HCC cells (data not shown).

## Vessel co-option during acquired resistance

Vessel co-option has been described in several human tumour types in organs such as the lung, liver, brain and, more recently, lymph nodes[10-12,33-35]. Since co-opted vessels are presumed to be 'normal' vessels and not susceptible to angiogenic inhibition, they have been realized as a potential mode of resistance to anti-angiogenic therapy[4]. The evidence in support of this relates to upfront, intrinsically resistant preclinical microsatellite glioblastoma tumours (in which vessels appeared 'normal')[36] and melanoma CNS micrometastasis (non-responding tumour vessels expressed CD34- and GLUT-1+ blood brain barrier markers)[37]. In many (but not all) cases human co-opted tumours are reported to be small or microscopic therefore vessel co-option could be a factor in the repeated failure of antiangiogenic drugs in phase III trials as adjuvant therapy including in HCC[5,38]. Here we demonstrated here that co-option occurs in large primary HCC tumours prior to treatment (at proportionately low levels) but its contribution intensified after sustained angiogenic inhibition. Stopping therapy at this point relieves the angiogenic inhibition and causes rapid tumour rebound.

## Tumour cell invasiveness as a mechanism for co-option

The molecular mechanisms contributing to vessel co-option are poorly understood and have been investigated only in angiogenesis inhibitor-naïve (non-treated) tumours[13]. We provide the mechanism that tumour cells acquire EMT-like genetic changes which facilitate tumour cell invasion into the host tissue and direct perfusion of the tumour by the liver. Anti-angiogenic therapy has been observed to increase local invasion and metastasis in pre-clinical studies[4,39,40] and resistant HCC cells have been reported to undergo an EMT during TKI treatment *in vitro* to become more invasive, metastatic and refractory[28]. The relevance of such findings have been unclear since pre-existing advanced metastatic disease was not observed to become more aggressive in patients treated with angiogenesis inhibitors[41]. Moreover the physiological relevance of EMT (ie. a change in cellular identity) during tumour progression is

controversial since evidence of complete EMT has not been observed in human carcinoma specimens[42,43]. Most tumour types do not lose epithelial characteristics and they more frequently invade and metastasize collectively as cell clusters or nests rather than as single cells[43,44]. We propose that the increased local invasiveness observed with anti-angiogenic therapy (which may not be accompanied by increased metastasis) is physiologically relevant because it permits tumours to co-opt pre-existing vessels. Thus the EMT-like molecular changes (e.g. increase in vimentin and ZEB2 expression without loss of epithelial adhesion proteins such as E-cadherin) may have allowed tumour cells to remain adhered and collectively invade without disseminating to distant sites[44].

Further, hypoxia could be an important generator of sorafenib resistance since it is a key inducer of EMT, various pro-survival pathways, myofibroblast proliferation and extracellular matrix remodeling[45,46]. Moreover loss of hypoxia could contribute to the reversal of resistance *in vitro* which may be independent of some of the direct effects sorafenib appears to have on cancer cell invasion when cells are grown in culture. The results of our miRNA screen do not preclude additional molecular drivers of vessel co-option and sorafenib resistance. For instance, serpin molecules are important for axonal guidance by inhibiting thrombin activation and limiting cell invasion and have recently been implicated in the migration and co-option of metastasized lung cancer cells along vessels in the brain[47].

## Up-regulation of pro-angiogenic factors without evidence of an angiogenic rebound effect

Third, we observed rebounds or 'spikes' in several pro-angiogenic growth factors despite a lack of a vascular rebound effect as assessed by global tumour perfusion, CD34+ or tumour embedded vessel density, or endothelial cell proliferation, while mice were maintained on therapy. Regions of enhanced perfusion during resistance corresponded with incorporated liver parenchyma rather than angiogenesis. Importantly, a commonly cited mechanism of anti-VEGF resistance is induction of VEGF-independent (alternate) compensatory angiogenic signaling[4,5] but our data show a discordance between the emergence

of angiogenic stimuli with angiogenesis. This offers an explanation for the difficulty in obtaining reliable biomarkers of patient response to anti-VEGF agents including sorafenib[48].

The up-regulated pro-angiogenic growth factors, particularly mouse and human VEGF and mOPN, could be due to a hypoxic compensatory response to re-establish a vascular supply via angiogenesis, which then fails as a result of VEGFR inhibition. VEGF also has known angiogenesis-independent functions, including stimulation of tumour cell or hepatocyte growth[49] and induction of tumour cell invasion through an autocrine loop[50]. Similar functions have been reported for OPN and induction of HCC tumour cell invasion[51]. Unlike the other factors, mouse Ang2 correlated positively with tumour-embedded (angiogenic) vessels but its levels tended to increase from the drug-sensitive phase to the co-option-driven resistant phase. These data are interesting because Ang2 has demonstrated both agonistic and antagonistic activity on its receptor Tie2 (which is involved in regulating vascular stability) depending on the cellular context such as the presence of VEGF[52]. Moreover, Ang2/Tie2 and VEGF/VEGFR2 signaling were implicated in the regulation of the co-option/angiogenic switch in a rat glioma model[53]. Further investigation into whether the above growth factors are either drivers or bystanders of the co-option process, is needed.

## Progression patterns during anti-VEGF resistance

Tumour perfusion patterns in patients that have acquired resistance to anti-angiogenic therapy have not been well characterized. In one report of 41 RCC patients progressing on anti-angiogenic TKIs after >250 days of initial therapeutic response, only 24.3% of patients experienced either a central fill-in pattern or a new enhancement in a non-enhancing mass which are suggestive of angiogenic rebound[7]. Similarly, a ‘flare-up’ enhancing progression on bevacizumab has been reported in 42% of glioma patients[9] while 18-30% of patients experience a non-enhancing, diffuse type of progression[9,54]. Such a

progression pattern could reflect vessel co-option, since similar tumours appear invasive[54], have reduced vascularity and contain 'normal' looking vessels[55].

Using conventional image processing, no induction in global perfusion was observed by ultrasound, but by local perfusion analysis we observed a major shift in the distribution of peak enhancement (PE) values, a pattern that persisted into Late Resistance time points. Wide PE distribution reflected primarily angiogenic and hemorrhagic tumour-embedded (CD34+) vessels and to a lesser extent HVs and large liver vessels. The shift in functional vessels matched the observed shift toward co-opted liver (primarily HV) vessels and the loss of functionality and depletion of TVs. Most importantly, the prevalence of a hyper-enhanced rim in resistant tumours corresponded to active areas of co-option rather than regions of angiogenic rebound, which might otherwise be assumed. Testing whether similar perfusion changes can be detected in cancer patients is important for future research, since it could potentially be used to guide anti-vascular treatment selection for cancer treatment.

## Additional implications and conclusions

In summary, increased local infiltration of cancer cells that facilitates vessel co-option may explain the reversible acquired resistance phenotype observed in tumours treated with TKIs. To prevent acquired resistance caused by vessel co-option new approaches such as targeting or delaying the cellular invasion that precedes co-option may be necessary. Drugs which target pro-invasive mediators such as c-met and VEGFRs simultaneously (e.g. cabozantanib) may be ideal for this. Anti-angiogenic therapy combined with chemotherapy also may mitigate the pro-invasive effects of anti-VEGFR2 treatment[56], although there is no standard chemotherapy in HCC. Modifications to the schedule of sorafenib administration could also exploit the reversible resistance phenotype. Future work should interrogate the molecular profile of co-opted vessels and assess whether they have unique properties distinguising them from normal host vessels which may reveal new molecular targets for anti-vascular therapy.

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# Chapter 4. Drug Rechallenge and Treatment Beyond Progression – Implications for Drug Resistance

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Attributions: The majority of research and writing, the design and conception of figures and topics discussed was performed by Elizabeth Kuczynski. The initial idea to produce this Review came from Robert Kerbel, Axel Grothey and Dan Sargent. Assistance with the conception and editing of this Review as well as some of the writing and research was performed by Dan Sargent, Axel Grothey and Robert Kerbel.

## ABSTRACT

The established dogma in oncology for managing recurrent or refractory disease dictates that therapy is changed at disease progression since the cancer is assumed to have become drug-resistant. Drug resistance, whether pre-existing or acquired, is largely thought to be a stable and heritable process, thus, reuse of therapeutic agents that have failed is generally contraindicated. Over the past few decades, clinical evidence has suggested a role for unstable, non-heritable mechanisms of acquired drug resistance pertaining to chemotherapy and targeted agents. There are many examples of circumstances where patients respond to reintroduction of the same therapy (drug rechallenge) after a drug holiday following disease relapse or progression during therapy. Additional, albeit limited, evidence suggests that in certain circumstances continuing a therapy beyond disease progression can also have antitumour activity. In this Review, we describe the anticancer agents used in these treatment strategies and discuss the potential mechanisms explaining the apparent tumour re-sensitization with reintroduced or continued therapy. The extensive number of malignancies and drugs that do not fit with the traditional treatment dogma warrants a more in-depth examination of the definitions of disease progression and drug resistance and the resulting implications for patient care.

## KEY POINTS

Reuse of the same anticancer therapy following disease progression is often considered to be futile due to drug resistance; however, many cancers show sensitivity to therapy reintroduction after disease progression.

- Spontaneous, reversible and epigenetic resistance mechanisms may explain the retreatment phenomenon; alternatively, a growing tumour may undergo progression on therapy in the absence of drug resistance
- Selection of drug-resistant clones is not necessarily a major contributor to response to therapy in many patients
- Definitions of drug resistance need to be re-evaluated; for example, disease progression based on RECIST criteria might be a poor indicator of drug resistance and when to change a course of treatment
- Applying transient drug-resistance mechanisms to clinical practice could offer advantages over traditional therapy regimens, including increased therapeutic options, reduced costs, and improvements in quality of life, without compromising efficacy

## INTRODUCTION

Historically, the most important factor to limit the success of systemic anticancer therapy in achieving cure or prolonged overall survival has been drug resistance. This has become apparent after using chemotherapy drugs for more than half a century, but continues to be as formidable a problem, if not more so, in the current era of molecularly targeted drugs and personalized medicine. There are two types of cancer drug resistance: intrinsic (also called innate or primary resistance) or acquired (also called evasive, adaptive, or secondary resistance). In this Review we will focus on acquired resistance, particularly with regard to the stability—or lack thereof—of the acquired drug-resistant phenotype.

In the clinic, resistant disease describes cancer that is found to have progressed since the time of treatment initiation. It is not uncommon to find that the term 'drug resistant' is used synonymously with 'progressive disease' when referring to a treated tumour. Once a patient develops acquired resistance to a given agent, the usual accepted strategy is to initiate a different therapy on resistant (refractory) disease using non-cross-resistant drugs. The underlying assumption is that previously used agents are obsolete, and it is on this premise that treatment guidelines (e.g. NCCN) for nearly all cancers are built. According to the classic Goldie–Coldman hypothesis of drug resistance[1], mutations are spontaneously acquired by the tumour over time leading to an accumulation of drug-resistant clones. Resistant variants in a heterogeneous tumour can be selected for in a Darwinian evolutionary process[2], or a quiescent subpopulation of intrinsically drug-resistant cancer stem cells may cause regrowth or spread of the tumour at progression[3]. A tumour that has progressed on therapy is assumed to have permanently changed, necessitating a different treatment plan.

The view that acquired drug resistance is almost always stable and irreversible stems from a number of reasons. First, many of the early pioneering studies of drug resistance undertook the selection and analysis of drug-resistant mutant cell clones in cell culture, usually using prolonged stepwise treatments of cell monolayers with ultimately very high concentrations of cytotoxic agents[4,5]. This can create a severe and sometimes artifactual selection pressure that is unlike the clinical situation. Such procedures led to the discovery of the multidrug-resistant (MDR) phenotype caused by overexpression of the P-glycoprotein drug efflux transporter in the 1970s[6]; however, the clinical relevance of MDR was questioned when multiple phase III trials of specific P-glycoprotein antagonists subsequently failed to show any efficacy[7]. A second reason was the discovery of defined mutations in genes encoding molecular drug targets, such as EGFR, BCR-ABL, or c-Kit, which have been found to explain acquired resistance to drugs such as the small-molecule tyrosine kinase inhibitors (TKIs) gefitinib, erlotinib, or imatinib[8,9]. Moreover, deep sequencing genomic studies that revealed the enormous extent of genetic heterogeneity of

human tumours has added to the perception of fixed pre-existing (or induced) gene mutations being primarily responsible for acquired drug resistance in cancer[10,11]. Finally, the act of permanently stopping a type of therapy at radiographic disease progression reinforces the belief of permanent drug resistance. Since stopping a treatment at the time of progression is the current dominant paradigm of clinical trial conduct, available data from clinical trials are routinely not able to provide any information that could challenge this notion.

Box 1: Historical and recent examples of drug rechallenge

Chemotherapy rechallenge dates back to the 1970s with the retreatment of combination chemotherapy in Hodgkin lymphoma[167], and multiple myeloma[168]. Early studies primarily reported on drug rechallenge in small-cell lung cancer, various leukaemias and following adjuvant treatment of breast cancer[117]. Presently, rechallenge-like regimens used for retreatment include anthracyclines and taxanes in adjuvant or metastatic breast cancer[150], platinum-based therapy in ovarian cancer[13], tamoxifen in oestrogen receptor-positive breast cancer[169], and diethylstilbesterone or maximum androgen blockade in androgen-independent prostate cancer[28,170,171]. Specific experiences with chemotherapy rechallenge have changed definitions of drug resistance to exclude relapses that occur after a prolonged period off therapy.

**Recent examples***

- Cytotoxic chemotherapy agents: docetaxel, irinotecan, oxaliplatin, temozolomide, trabectedin
- Kinase inhibitors: erlotinib (EGFR), gefitinib (EGFR), imatinib (BCR-ABL/c-Kit), sorafenib (VEGFR), sunitinib (VEGFR)
- Monoclonal antibodies: alemtuzumab (CD52), bevacizumab (VEGFR), cetuximab (EGFR), rituximab (CD20), trastuzumab (HER2)
- Proteasome inhibitors: bortezomib

*Drug molecular targets are in brackets.

## UNSTABLE, NON-HERITABLE DRUG RESISTANCE

There are many examples from the clinic that compete with the archetype of managing recurrent or refractory disease in that patients' tumours can be sensitive to the agent(s) they had originally progressed on (Box 1, Figure 4.1). As a first example, a patient experiences disease progression (relapses) following discontinuation of therapy and is rechallenged with the same therapy, typically with the same dose and regimen, after a treatment-free interval. Second, a patient experiences disease progression during a course of therapy and is rechallenged with the same therapy following an intervening therapy. Third, a patient experiences disease progression during a course of therapy, but continues the therapy —typically in combination with a new agent— without stopping.

These treatment strategies have demonstrated activity in a wide variety of malignancies using conventional chemotherapeutic drugs as well as many of the newer and older (hormonal) molecular targeted therapies. Drug rechallenge and continuation of treatment following progression on therapy are strategies that have emerged over the past decade. Rechallenging a tumour that has relapsed off therapy is an old concept (Box 1). This topic was last reviewed over a decade ago, with the conclusion that many seemingly drug-resistant cancers, somehow, may not be resistant after all[12]. In a few specific cases for treatment of ovarian, colorectal and small-cell lung carcinoma, this concept has led to the development of new, more flexible definitions of drug resistance. Relapses may be termed 'sensitive' or 'partially sensitive' rather than 'resistant' if the treatment-free interval from therapy discontinuation to relapse is of long (or intermediate) duration, since the longer the time to progression, the greater the chance of a response to retreatment[13-15]. These definitions also add confusion to the debate on what constitutes true drug resistance.

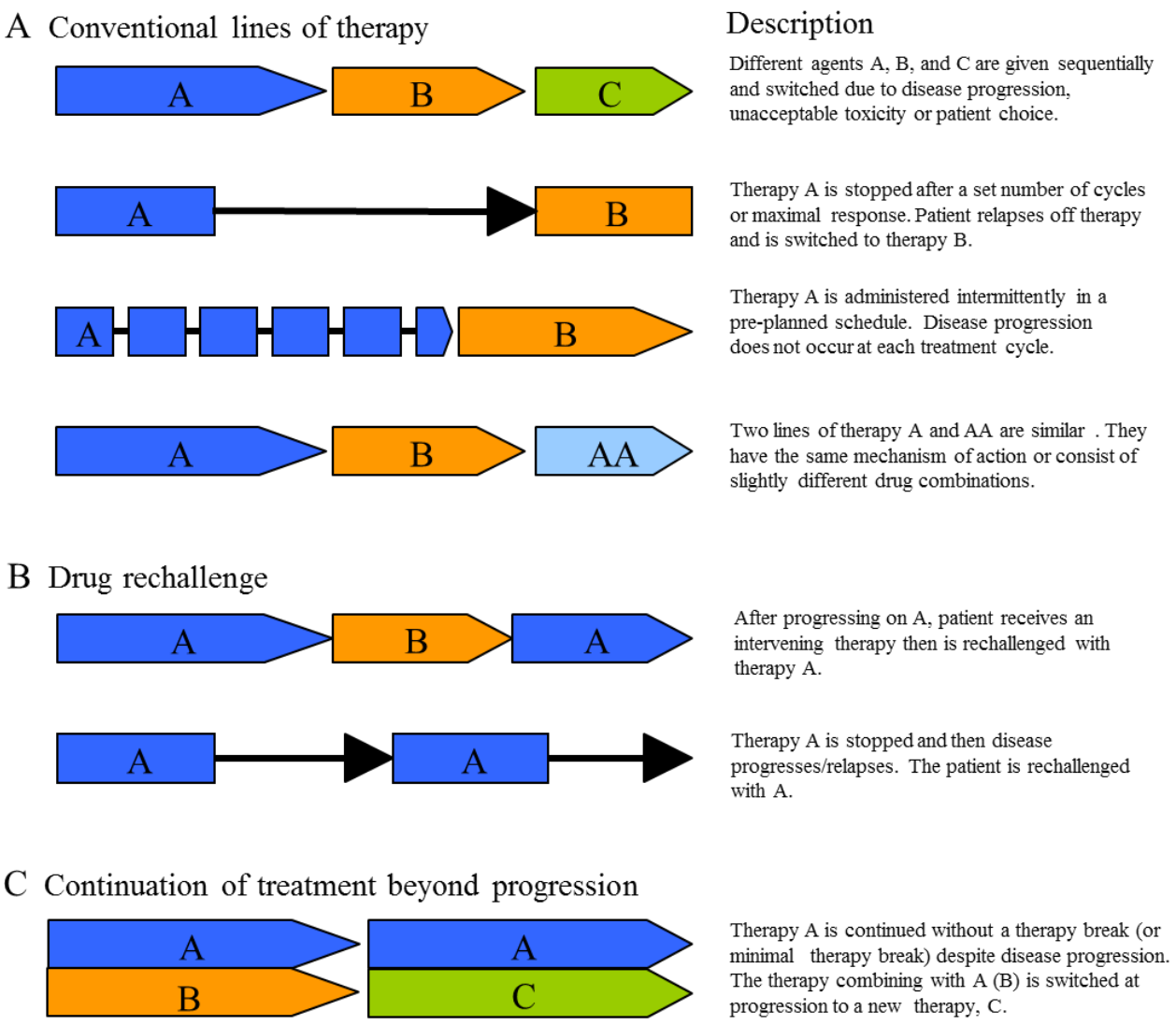


**Figure 4.1** Conventional and nonconventional (drug rechallenge and treatment beyond progression) therapy regimens in medical oncology. **A**. Typical sequences of therapy in relation to disease progression. **B**. Two major types of drug rechallenge. In drug rechallenge, treatment with a previously used agent(s) is repeated despite prior failure of the treatment, consisting of disease progression on therapy or after discontinuation of the therapy. **C**. In treatment beyond progression a therapy is continued with no break or a minimal break at disease progression. Arrowheads: progressive disease. Black lines/arrows: drug-free interval.

Although the treatment scenarios described above are biologically and clinically quite distinct, their anti-tumour activity implies that many ostensibly resistant tumours were either not resistant at initial progression, or that the resistant phenotype was transient. Thus, heritable mechanisms driving drug resistance and response to future therapy are ruled out. Indeed, recent evidence suggests that many characteristics of tumours, such as persistence of clones, altered tumour dynamics, tumour heterogeneity and response to therapy, may be derived from genomically similar clones and not necessarily by mutations[16]. Despite its apparent prevalence, unstable or transient resistance has received little attention as a major concept in medical oncology.

In this Review, we discuss the therapeutic agents from the past decade that have been used to rechallenge patients with cancer who have progressed during therapy or at relapse, or that have been continued in patients beyond disease progression. In many cases, these strategies are routinely being used in the clinic or incorporated into the standard of care. The differences between each retreatment strategy and its implication for drug resistance, and possible mechanisms of non-heritable or reversible drug resistance are examined. Finally, the implications that unstable acquired resistance may have for patient care, clinical benefit, clinical practice, and cost of cancer therapy are discussed. For example, if a patient who has progressed on therapy has merely developed a transient insensitivity to that agent, the possibility of retreatment increases the number of therapeutic options available, raising the issue of how to identify true resistance and establish when a drug has become futile. The abundant examples that counter the long-standing convention of changing therapies at traditional disease progression should not be dismissed as exceptional cases. A re-evaluation of the definition of drug resistance, as well as the standard treatment dogma in oncology, is warranted.

## RECHALLENGE AFTER PROGRESSION OFF THERAPY

Most rechallenge studies are performed in patients experiencing disease progression off therapy and likely do not constitute rechallenge of resistant disease. The durable responses of several months to years in length achieved by retreatment therapy indicate a minor role for acquired permanent drug resistance in these settings. Drug rechallenge can be assessed by observing a prolonged time to secondary progression or positive anti-tumour response following therapy reintroduction, and by comparing these to initial drug exposure within the same patient or patient cohort (Figure 4.2). Some important examples of drug rechallenge are discussed below.

### Oxaliplatin-based chemotherapy in CRC

Combining 5-fluorouracil (5-FU) and leucovorin with newer agents such as oxaliplatin (FOLFOX), irinotecan (FOLFIRI), or the monoclonal antibodies bevacizumab or cetuximab has led to an increase in survival in patients with advanced colorectal carcinoma (CRC) from 12 months to several years[17,18]. Consequently, incorporating chemotherapy-free intervals into treatment algorithms has become important for the management of patient quality of life, particularly with regard to oxaliplatin treatment which may cause an accumulation of neurotoxicity[19]. Retrospective data indicate a benefit of interrupting FOLFOX therapy and reintroducing it at relapse, typically with a different regimen, with a high rate of response or disease stabilization at reintroduction[19,20]. Compared to other follow-up therapies, rechallenge with FOLFOXIRI (FOLFOX plus irinotecan) was shown to yield a significantly longer progression-free survival (PFS) (8.2 months rechallenge versus 6.3 months for other therapies, p=0.003) and overall survival (OS) (19.3 vs. 14.0 months, respectively, p=0.02) [21].

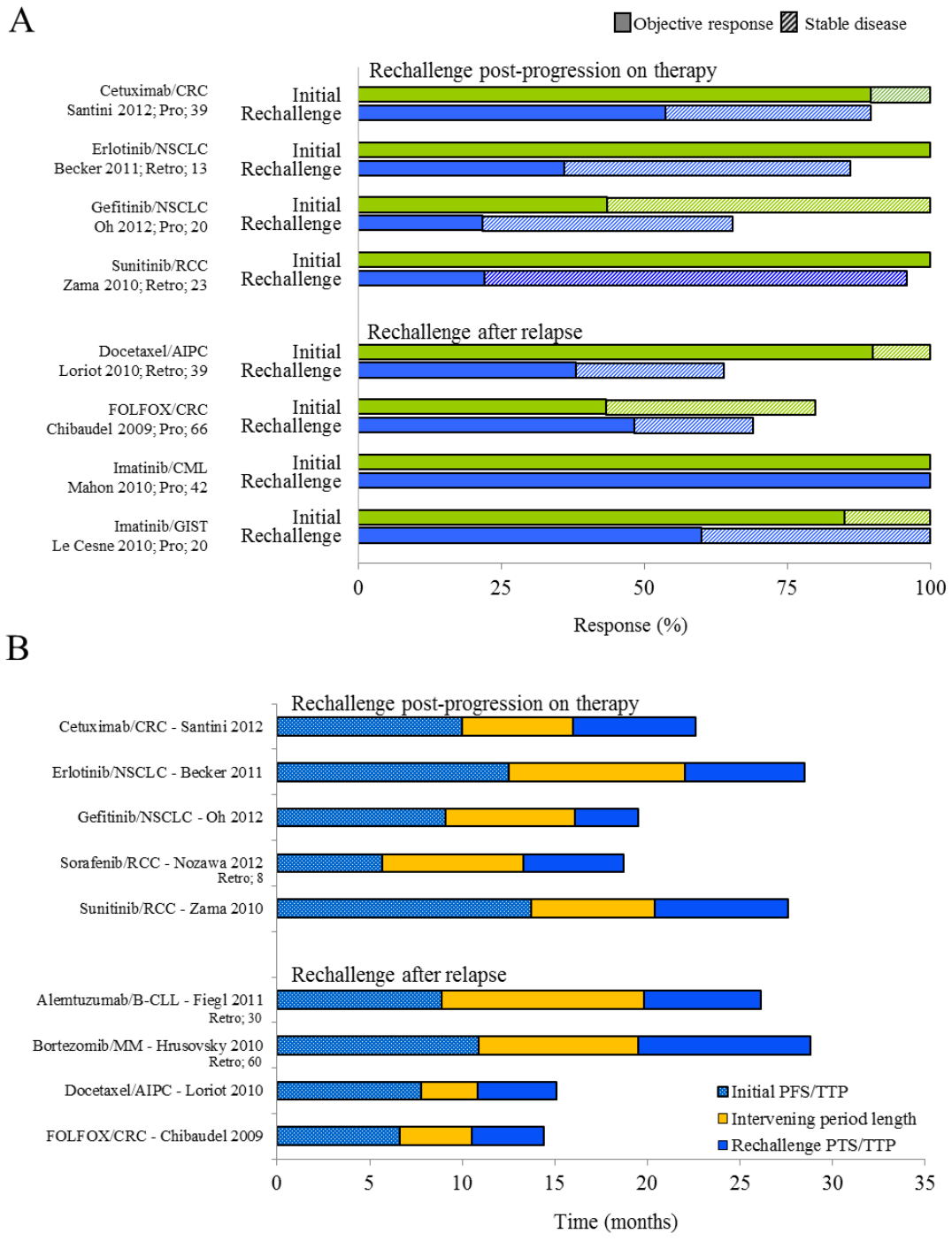


**Figure 4.2** Efficacy of drug rechallenge following disease progression on or off therapy. **A**. Examples of response rates at initial treatment and rechallenge. Tumour control rates are good, although generally weaker at rechallenge, and fewer objective responses are achieved. **B**. Progression-free survival (PFS)/time to progression (TTP) at initial therapy and at rechallenge in relation to the length of treatment-free interval. The rate of disease progression at

rechallenge is favourable, although shorter at rechallenge. A longer treatment-free interval is often related to the rechallenge PFS/TTP. Treatment-free interval consists of an intervening treatment if rechallenge post-progression on therapy. Numbers in bold indicates the number of patients rechallenged. Note: in Mahon 2010, rechallenge ORR includes 62% patients with complete molecular response and 38% patients with declining BCR-ABL transcripts. Loriot 2010, ORR = PSA decline ≥50%, and at rechallenge SD= PSA decline between 30 and 50%. Abbreviations: AIPC, androgen-independent prostate cancer; B-CLL, B-cell chronic lymphocytic leukaemia; CRC, colorectal carcinoma; GIST, gastrointestinal stromal tumour; MM, multiple myeloma; NSCLC, non-small-cell lung carcinoma; Pro, prospective study; PFS, progression-free survival; RCC, renal cell carcinoma; Retro, retrospective study; TTP, time to progression[24,34,41,44,58,67,69,79,81,148,153].

Chemotherapy-free intervals have been shown to result in improved efficacy in comparison with continuous treatment using well established chemotherapy regimens and drugs in patients with CRC[15,22]. Rechallenge strategies with newer agents have also been studied in patients with metastatic CRC in prospective randomized phase III (OPTIMOX1) and phase II (OPTIMOX2) trials[23,24]. In OPTIMOX1, 620 previously untreated patients received either continuous FOLFOX4 administered every two weeks until progression (arm A), or six cycles of FOLFOX7 followed by a simplified regimen of maintenance 5-FU and leucovorin for 12 cycles and then reintroduction of FOLFOX7 (arm B). FOLFOX7 is a more dose-intensive regimen of oxaliplatin than FOLFOX4. Oxaliplatin was reintroduced in only 40.1% of patients: patients who experienced PD prior to FOLFOX7 reintroduction (89 patients) experienced a lower response rate and rate disease stabilization (6.7% and 42.7%) than patients who did not first experience PD (33 patients; 24.2% vs. 54.4%, respectively) [23]. OS was not found to be significantly better in the FOLFOX reintroduction arm (21.2 months vs. 19.3 months in the FOLFOX4 arm; p=0.49), but after accounting for biases encountered in the original trial[23], a subsequent analysis found that reintroduction of oxaliplatin had an independent significant positive impact on OS (HR=0.56, p=0.009)[25]. In OPTIMOX2, 202 patients with previously untreated metastatic CRC received six cycles of modified FOLFOX7 and were randomized to either completely stop chemotherapy or receive maintenance chemotherapy without oxaliplatin until disease progression. At progression, another six cycles of

FOLFOX7 were reintroduced in both treatment arms. The chemotherapy-free interval had a negative impact on PFS (6.6 vs. 8.6 months in the maintenance arm, p=0.0017) and OS (19.5 vs. 23.8 months, respectively, p=0.42)[24]. An analysis of data from both trials found that a prolonged interval between courses of FOLFOX or a longer initial PFS may be predictive of the efficacy of this strategy, with median survival from reintroduction at 8.9, 16.6 and 22.1 months if the FOLFOX-free interval was <6 months, 6-12 months or ≥12 months, respectively (p<0.0001)[26]. To conclude whether continuous or intermittent therapy is superior, the phase III MRC COIN trial (a three-armed trial in previously untreated patients) was conducted, and entailed one continuous oxaliplatin and 5-FU or capecitabine arm as well as an intermittent therapy arm in which patients were rechallenged after progression following 12 weeks of initial chemotherapy (815 patients per group). Of 268 patients that were rechallenged and assessable, 33% of patients had a PR, and 38% SD. This trial failed to show non-inferiority with rechallenge compared to continuous oxaliplatin-based chemotherapy (OS 14.4 vs. 15.8 months, respectively) but rechallenge did improve quality of life, reduced time on chemotherapy and hospital visits (of note pain was worse in the intermittent arm)[27]. Overall, oxaliplatin rechallenge demonstrates similar efficacy to continuous regimens, but rechallenge may be preferred for the long-term management of CRC given its improved tolerability[19].

## Docetaxel in prostate cancer

Docetaxel chemotherapy is standard first-line therapy in metastatic castration-resistant, androgen-independent prostate cancer (AIPC). The possibility of intermittent therapy has been examined to alleviate excessive toxicity and avoid unnecessary treatment in responding patients, a strategy shown to be successful in hormonal therapy for prostate cancer[28]. Multiple retrospective studies have shown that incorporating a therapy break may lead to objective responses and, occasionally, improvements in quality of life if patients are rechallenged after relapse[29-33]. In the largest of these studies, 48% of initially responding patients (*n*=50) achieved a biochemical response (≥50% decline in prostate specific antigen [PSA]) at re-exposure, which is comparable to other therapies after docetaxel failure[31]. Moreover, an

initial biochemical response[30] or prolonged docetaxel-free period[34] have been found to correlate significantly with PFS or overall survival at rechallenge. Additionally, greater than two rechallenges after subsequent relapses also appear to induce PSA responses in certain patients[30,35,36].

Two prospective trials have assessed the efficacy of rechallenge following a profound initial response to docetaxel[35,37]. In the ASCENT phase III clinical trial comparing calcitriol plus docetaxel to docetaxel alone, patients with progressive disease were later re-exposed to docetaxel therapy[35]. Of 36 patients rechallenged after one drug holiday, 45.5% had a reduction of PSA ≥50% and 45.5% had stable disease. Quality of life improvements were also noted in these patients, although rechallenged patients had more favourable prognoses than patients that were not rechallenged. In a multicentre phase II trial, 45 patients with AIPC who initially responded to docetaxel and then progressed after a period of biochemical remission of at least 5 months were retreated with docetaxel[37]. A partial biochemical response (defined as >50% PSA decline) was observed in 24.5% of patients and minor PSA reductions (>25%-49% PSA decline) or stable disease (<25% PSA decrease or increase) was observed in 22.2% of patients. Retreatment with docetaxel has become standard in patients with AIPC since alternative treatment strategies have not been defined; however, an ongoing phase III trial will help to establish whether continuous or intermittent docetaxel regimens are superior[38].

## Imatinib in GIST and CML

First-line imatinib, an oral TKI targeting c-Kit, PDGFRα and BCR–ABL, is the standard of care for patients with advanced-stage gastrointestinal stromal tumours (GIST) and is normally given long-term and continuously. Most patients with controlled GIST who discontinue imatinib rapidly experience disease progression[39]. The phase III BFR14 trial was conducted to determine the optimum duration of imatinib therapy and whether introducing therapy breaks influenced the onset of acquired resistance[40]. Patients with non-progressing GIST after 1, 3 or 5 years of imatinib treatment were randomized to either

continue or discontinue the drug. Following re-introduction of imatinib at disease progression patients regained tumour control. This was observed in 92% (32 patients) of patients previously treated for 1 year with imatinib, and in 100% of patients previously treated for 3 or 5 years (25 and 14 patients, respectively) [39,41,42]. There was no significant difference in overall survival or rates of progression on therapy (that is, development of resistance) between discontinuation and continuous groups at the first two randomizations[39,41]. Interestingly, regardless of the length of initial treatment, most patients progressed off therapy at the same rate, although patients who progressed rapidly after imatinib discontinuation had the poorest prognosis and achieved progression during imatinib rechallenge sooner (2 year PFS was 30%, 62% and 75% for patients who relapse in the first 6 months after discontinuation vs. between 6 and 12 months vs. after 12 months, respectively)[40]. Results from this trial also suggest that patients experiencing stronger initial responses to imatinib have a longer time to relapse after therapy discontinuation; however patients experiencing a complete response after long duration of treatment still have residual persistent sensitive tumour cells[40]. Similar findings for rechallenge after adjuvant imatinib suggest that recurrent disease is imatinib-sensitive and prior exposure does not limit the efficacy of imatinib[43].

Imatinib is also used to treat chronic myelocytic leukaemia (CML), through inhibition of BCR–ABL, and complete remissions are not uncommon. Disease recurs in a subset of patients with CML who discontinue imatinib following periods of durable remission[44]. The results of a phase II trial (TWISTER) which followed 40 CML patients with sustained undetectable minimal residual disease for 2 years (based on quantitative PCR of BCR-ABL transcripts) have recently been reported[45]. Approximately 40% of patients remained in deep remission for 24 months following imatinib discontinuation. If relapses occurred they mostly occurred within the first four months after therapy discontinuation, and all patients (22) regained undetectable minimal residual disease status at imatinib reintroduction upon early detection of relapse. Surprisingly, the BCR-ABL DNA remained stable without mutation at relapse. All five

patients that relapsed within 5 months regained a complete molecular response when rechallenged with imatinib. In another small study of 26 patients who discontinued imatinib after achieving complete remission, all 23 patients who relapsed and resumed imatinib treatment achieved a complete molecular or cytogenetic response of prolonged duration[46]. The largest study that has investigated imatinib rechallenge is the multicentre phase II STIM (Stop Imatinib) trial[44]. Of 69 patients with CML who discontinued imatinib after 2 years, 42 patients relapsed. All the relapsed patients responded to imatinib reintroduction, with prolonged complete molecular remission observed in 26 patients, and declining BCR–ABL levels seen in 16 patients[44]. The finding that both patients with GIST and CML respond remarkably well to reintroduction of imatinib suggests that permanent acquired may not occur at significant levels in all cases. Of note, the duration and intensity of responses seen with imatinib treatment of GIST and CML are not typical of other targeted therapies in other diseases. Despite the impressive tumour responses seen with imatinib reintroduction in both malignancies, imatinib discontinuation is not recommended if disease control is achieved unless patients experience significant toxicity[39,44].

## Temozolomide in glioblastoma

The alkylating agent temozolomide is used as a front-line therapy in combination with radiotherapy and as salvage therapy in high-grade recurrent malignant glioma. Temozolomide has been tested in various rechallenge settings—following disease progression on therapy and following relapse after temozolomide discontinuation—since there is no consensus on subsequent therapies[47]. The focus of rechallenge has been on using an alternative dosing strategy in an effort to overcome initial temozolomide resistance. Depleting methylguanine-DNA methyltransferase (MGMT) levels or inhibiting angiogenesis are both hypothesized to be affected through more protracted and dose-intensified regimens following an initial standard schedule[48,49]. Retreatment with different temozolomide schedules is well-tolerated and response rates seem to be comparable to other therapies[47,49-52]. Switching to a dose-intensified continuous 50 mg/m$^2$ temozolomide regimen at progression immediately or following a drug-free period from

conventional 150-200 mg/m$^2$ 5-day temozolomide both appear to be active strategies. Perry *et al*.[49] observed a clinical benefit rate of 47% and 6-month PFS of 17% with immediate switching in 21 patients and, in 14 patients relapsing after adjuvant or radiation plus concomitant temozolomide, an 79% clinical benefit rate and 6-month PFS of 57% was observed. In a retrospective study of patients with recurrent glioma rechallenged with the same or one of various different regimens of temozolomide (mostly dose-intensified), PFS at 6 months was 57.9% in patients with anaplastic glioma (19 patients) and 28.6% in patients with glioblastoma multiforme (28 patients) who had relapsed after a temozolomide free interval[47]. PFS-6 in patients that had been rechallenged after having failed on temozolomide (without a break) was 16.7% (6 patients) and 26.3% (19 patients), respectively[47].

Small prospective trials have demonstrated successful outcomes when drug rechallenge of different temozolomide regimens were used, even in patients with poor prognosis[53,54] and in patients whose tumours expressed MGMT or had unmethylated *MGMT* promoters[54,55]. The largest of these trials, the RESCUE study, prospectively stratified 120 patients with recurrent glioma previously on 5-day adjuvant temozolomide, into groups for 50 mg/m$^2$ temozolomide treatment based on the type of progression, including glioblastoma multiforme patients following progression on adjuvant temozolomide (early or extended progression) or rechallenge after a treatment free interval of more than 2 months[55]. Patients rechallenged with daily temozolomide after a prolonged treatment-free interval benefited the most from the new schedule compared to those who progressed earlier (PFS 3.7 months for the treatment-free interval group, 3.6 months early and 1.8 months late progression). The ongoing DIRECTOR phase II trial will further compare two dosing regimens of temozolomide in patients with relapsed or progressive glioma[56]. It has been suggested that repeating the same regimen and administering therapy breaks is not necessary and may cause lost time[54] but the necessity of changing the regimen of temozolomide at progression has not yet been tested.

## RECHALLENGE AFTER PROGRESSION ON THERAPY

Disease progression on therapy represents a newer setting for drug rechallenge, likely due to the increased use of molecular targeted agents that enable extended treatment duration (Figure 4.2), and involves retreatment of what may be considered as truly drug-resistant disease.

### Cetuximab-based therapy in colorectal cancer

Cetuximab is a monoclonal antibody that binds to the extracellular domain of EGFR and is used to treat metastatic CRC. A case series of four patients with CRC showed that rechallenge with the same cetuximab-containing therapy was effective following the development of progressive disease on therapy and treatment with an intervening therapy[57]. A single arm phase II multicentre trial in patients with *KRAS* wildtype colorectal tumours was conducted to examine the benefit of cetuximab rechallenge after progression on cetuximab-based therapy. This strategy was hypothesized to be effective because *KRAS* mutation status was not expected to change during treatment and therefore impact the efficacy of later exposures[58]. Indeed, of 39 patients rechallenged following progression on intervening therapy, 53.8% achieved an objective response and 35.9% had stable disease and 51.2% of patients achieved the same or better response as initial treatment. Stable disease of greater than 6 months and detection of a partial response at initial therapy were predictive of clinical benefit. The authors suggested that intervening therapy caused an increase in the proportion of sensitive tumour cells prior to cetuximab re-exposure[57,58].

#### EGFR inhibitors in NSCLC

Gefitinib is a selective EGFR oral TKI given continuously as monotherapy to patients with non-small-cell lung cancer (NSCLC). Although FDA approval was withdrawn for new users in 2005, following the approval of erlotinib, it is still widely used in Europe and Asia[59]. Patients with NSCLC have been shown to retain sensitivity to EGFR TKIs when they are switched to erlotinib after gefitinib

failure[60]. However, numerous case reports suggest that retained sensitivity may also occur when gefitinib is reused after disease progression[61-63]. In a retrospective analysis of 27 patients with NSCLC who showed an initial response to gefitinib, rechallenge with the drug in five evaluable patients resulted in a partial response in one patient and stable disease in three patients[64]. In another study of 20 patients with NSCLC, a partial response was observed in 16 patients and stable disease in four patients after initial treatment with gefitinib. Re-exposure of all patients (following a median of 7.2 months of cytotoxic therapy) led to a partial response in five patients and stable disease in eight patients[65]. A few small single-arm phase II trials have investigated the efficacy of gefitinib rechallenge[66,67]. In a study of 16 patients with advanced NSCLC who initially responded to gefitinib, retreatment with gefitinib did not shrink tumours; however, it stabilized disease in seven patients, and in four of these patients this response lasted for 6 months or longer[66]. In another trial of 23 patients with NSCLC, 43.5% of patients had a partial response and 56.5% had stable disease after initial treatment with gefitinib[67]. Following rechallenge, a partial response was observed in 21.7% of patients and 43.5% had stable disease. Patients who initially exhibited a partial response had a better response to re-exposure and a longer time to disease progression (TTP) than those with stable disease (median TTP of 109 days versus 42 days, p=0.010) but had no significant improvement in OS (337 days vs. 372 days, respectively, p=0.685). Pre-existing acquired *EGFR* mutations were not associated with response to rechallenge[67].

Erlotinib has also demonstrated success when used in a rechallenge regimen[68-70]. In medical reports of 14 patients with stage IV NSCLC who initially responded and then progressed on erlotinib, remarkably, a partial response was observed in 36% of patients and stable disease in 50% following erlotinib retreatment, with responses observed even in patients with T790M *EGFR* mutations (8 of these patients received erlotinib monotherapy at both exposures)[69]. The median interval between TKI exposures was 9.5 months and the initial and rechallenge median PFS were 12.5 and 6.5 months**.** The EGFR TKI-free period has been proposed to enable regrowth of EGFR-sensitive cells and actually benefit patients

with NSCLC that initially responded to the TKI[68,69]. At this time, only retrospective data suggests a survival benefit of rechallenge with gefitinib versus cytotoxic therapy[71], but rechallenge regimens have not been prospectively compared with non-rechallenge regimens. Retreatment seems to be a promising opportunity in NSCLC, particularly since subsequent lines of therapy are undefined.

**Imatinib in GIST**

If early discontinuation and subsequent rechallenge of imatinib in GIST does not cause significant expansion of drug-resistant clones, how might responses differ if patient's disease initially progressed while taking imatinib? In a small retrospective study of 26 patients with imatinib-refractory or intolerant GIST, re-induction of stable disease was observed with imatinib in 21% of 14 patients, and OS was non-significantly improved compared to 12 patients treated with best supportive care (22 months vs. 4 months, p=0.059)[72]. A review of medical records for 223 patients with GIST resistant to first-line imatinib and second-line sunitinib revealed that third-line treatment with an alternate TKI, nilotinib (67 patients) or sorafenib (55 patients) provided the greatest overall survival (11.8 months and 10.4 months, respectively)[73]. However, after adjusting for prognostic factors in a multivariate analysis, rechallenge with imatinib (40 patients) was associated with improved OS compared to best supportive care (18 patients; OS 7.5 months versus 2.4 months, HR 0.2, p=0.001). The efficacy of retreating resistant GIST has been confirmed by prospective data[74]. In the phase III RIGHT study, 81 patients with GIST refractory to both imatinib and sunitinib were randomized to receive either placebo or imatinib[74]. Compared to the placebo group, patients rechallenged with imatinib had a significantly greater PFS (1.8 months versus 0.9 months, p=0.002) and disease control rate (32% versus 5%, p=0.003) at 12 weeks. A modest but not statistically significant improvement in overall survival was observed with imatinib compared to placebo (8.2 months versus 7.5 months); this lack of significance might be because 92% of patients in the placebo arm crossed over to the imatinib arm after disease progression. Although subsequent responses to imatinib are weaker if the patient had initial disease progression on therapy rather than off therapy[40,74], a clinically meaningful

proportion of cells continue to remain imatinib-sensitive at progression. Guidelines from the National Comprehensive Cancer Network (NCCN) recommend rechallenge with imatinib in GIST, if tolerable, as late-line palliative therapy after failure on TKIs[75,76].

## VEGFR kinase inhibitors in RCC

Multiple antiangiogenic TKIs that primarily target VEGFR and PDGFR, such as sunitinib, sorafenib, pazopanib and axitinib, are approved as monotherapy for treatment of metastatic renal cell carcinoma (RCC). After progression on therapy, patients with RCC are frequently switched to an alternative antiangiogenic TKI, mainly because of the abundance of drug therapies. Surprisingly, this strategy is effective and suggests an absence of complete cross-resistance between TKIs[77]. A similar strategy has also shown to have some activity in patients with hepatocellular carcinoma[78]. Zama *et al.*[79] retrospectively examined whether rechallenge with the same agent could have a similar effect in patients with RCC. 23 patients with metastatic RCC who had disease progression after an initial response to sunitinib (PFS 13.7 months) were rechallenged after intervening therapy (primarily sorafenib with or without bevacizumab or an mTOR inhibitor) and experienced a second PFS of 7.2 months. Although retreatment generally yielded fewer objective responses (p=0.006) and a shorter PFS (0.04), PFS was significantly longer in patients with more than 6 months between exposures compared to patients with an interval between sunitinib treatments of 6 months or less (16.5 months versus 6.0 months, respectively, p=0.03). In a second study, 13 patients with metastatic RCC were re-exposed to sunitinib after first-line sunitinib and second-line treatment with mTOR inhibitors (temsirolimus or everolimus)[80]. In this study, 92% of patients achieved either a partial response or stable disease with sunitinib rechallenge, and failure to respond to an mTOR inhibitor was not associated with the outcome of rechallenge. In another study of patients with metastatic RCC, stable disease was observed in six out of eight patients who originally discontinued sorafenib because of progressive disease and were then retreated with the same drug[81]. In these studies, antiangiogenic TKI rechallenge seems to have activity in RCC—and possibly in other

malignancies, as observed in GIST case studies[82,83]— suggesting that resistance to sunitinib or sorafenib might be transient, at least in some individuals[80].

## CONTINUATION OF TREATMENT BEYOND PROGRESSION

Randomized studies in which patients either continue or discontinue an agent after disease progression are essential to establish whether continuing an agent beyond progression is efficacious. Whereas patients who are rechallenged serve as their own controls (initial versus subsequent response to treatment), patients who continue a treatment may have very different characteristics compared to those who discontinue treatment. For example, a patient with a minor progression (such as a 20% increase in the diameter of a small nodule) is more likely to continue and benefit from therapy than a patient who has progressed rapidly with new extensive metastases. Randomized studies of treatment beyond progression (Figure 4.3) are discussed below.

### Trastuzumab in breast cancer

Trastuzumab is a monoclonal anti-HER2 antibody approved for the treatment of *HER2*-overexpressing metastatic breast cancer in both the adjuvant and metastatic treatment settings. The safety and ability to combine trastuzumab with other agents has led to the practice of continuing trastuzumab treatment beyond progression while switching other lines of chemotherapy. Since 2004, a number of observational or retrospective studies have indicated that continuing trastuzumab treatment beyond disease progression is superior in terms of response rates, PFS and overall survival than discontinuing trastuzumab and switching to chemotherapy[84-87]. For example, a large observational study observed that within 177 patients with metastatic breast cancer receiving first line trastuzumab, overall survival measured from the time of first progression was 4.6 months in patients who discontinued trastuzumab and

21.3 months if treatment was continued ($p<0.001$)[88]. Prospective data from randomized phase III trials investigating the continuation of trastuzumab treatment beyond disease progression have been reported[89,90]. The German Breast Group 26/Breast International Group 03-05 study demonstrated an improvement in response rate and TTP when patients with metastatic breast cancer who had progressed on trastuzumab-based therapies continued the combination of capecitabine plus trastuzumab (78 patients; TBP) versus capecitabine alone (78 patients) at disease progression (response rate, 48.1% versus 27.0%, OR 2.50 p=0.0115 and TTP, 8.2 months versus 5.6 months, HR 0.69, p=0.0338, respectively[89]**.** Although underpowered, this trial demonstrated a trend toward improving survival with the TBP regimen. In another phase III trial of heavily pretreated patients on trastuzumab therapy with metastatic breast cancer, the combination of lapatinib plus trastuzumab compared to single-agent lapatinib significantly improved PFS (HR 0.73, p=0.008) and the clinical benefit rate (24.7% vs. 12.4%, p=0.01)[90]. Further analysis of the results from this trial revealed that continuing trastuzumab beyond progression significantly improved overall survival by 4.5 months (OS 51.6 weeks TBP vs. 39.0 weeks lapatinib alone; HR 0.74, p=0.026)[91]. Results with this drug combination suggest that enhanced blockade of HER2 can overcome resistance, and that trastuzumab still has activity beyond progression. Ongoing trials are exploring the use of trastuzumab in successive treatments and current NCCN guidelines recommend continued HER2 suppression after disease progression[92].

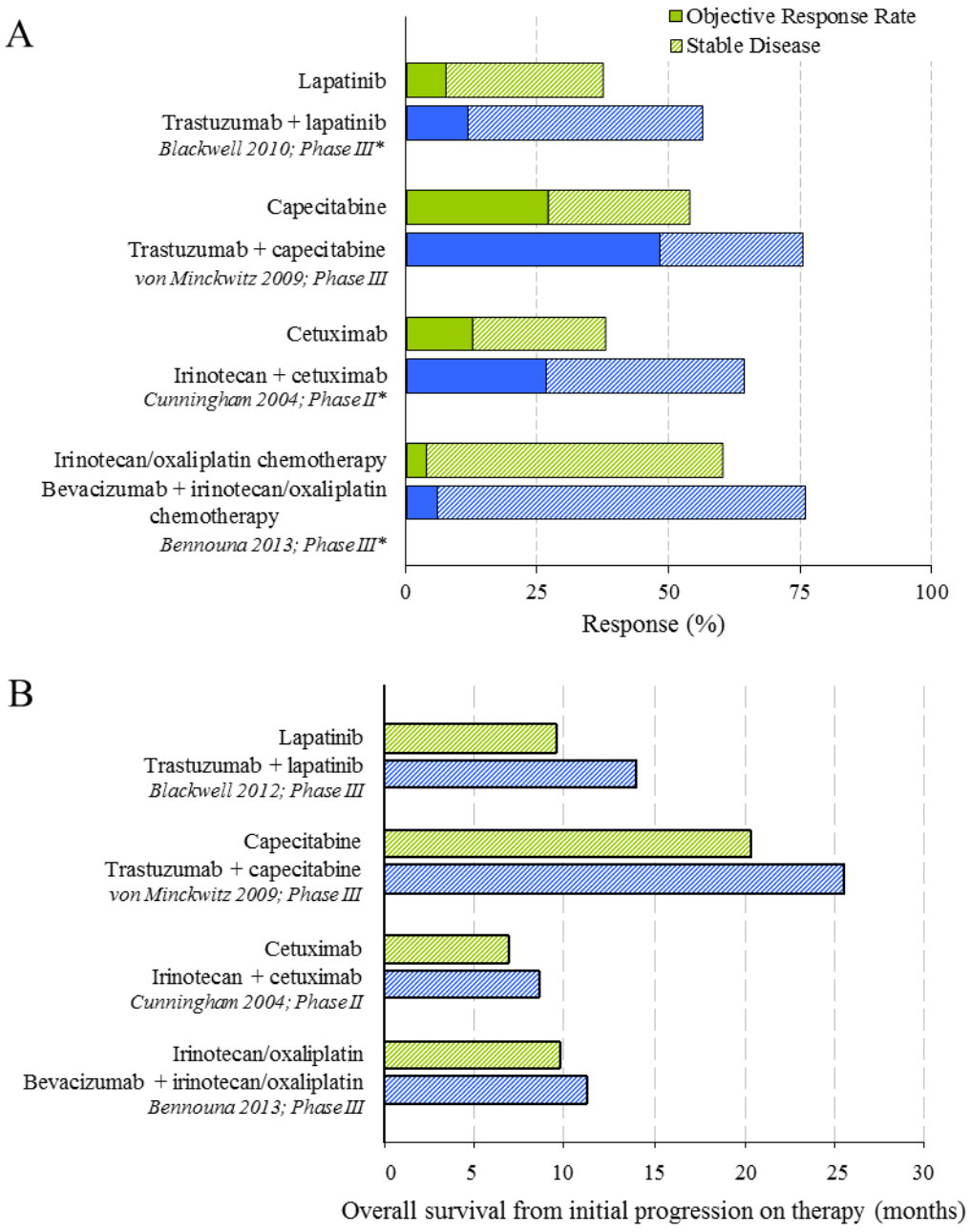


**Figure 4.3** Efficacy of treatment beyond progression in four randomized phase II or III clinical trials. **A**. Objective response rate and stable disease (%) and **B**. median overall survival following disease progression on trastuzumab, irinotecan and bevacizumab-based chemotherapy when treatment is continued (or not) beyond progression. Continuing a therapy is associated with significant improvements in survival and objective tumour responses[89,90,91,94,97].

## Bevacizumab in CRC

Bevacizumab improves survival in metastatic CRC when used in the first-line and second-line setting in combination with chemotherapy. Two large observational studies have seen a benefit when bevacizumab treatment is continued beyond disease progression. In the BRiTE study, of 1,445 previously untreated CRC patients experiencing PD on a bevacizumab-containing regimen, continuation of bevacizumab treatment beyond progression (642 patients) was significantly associated with an improvement in overall survival of 31.8 months versus 19.9 months in patients who discontinued only the bevacizumab (531 patients), or 12.6 months if no treatment at all (253 patients; HR for beyond progression vs. no beyond progression 0.48, $p<0.001$)[18]. These results were confirmed in the ARIES study which enrolled 1,546 patients receiving first or second line bevacizumab plus chemotherapy. In first line patients experiencing PD, 539 patients continuing bevacizumab beyond progression had a median beyond progression survival of 16.3 months, vs. 8.5 months if only the bevacizumab was discontinued (417 patients) vs. 5.2 months if all therapy was discontinued (127 patients). Continuation beyond progression was independently associated with improved survival beyond progression (HR 0.41, $p<0.001$)[93]. However, due to their observational design, it is likely that these studies were biased. The concept of treatment beyond progression has been validated in the randomized phase III Treatment Across Multiple Lines trial[94]. In this study, 820 patients with metastatic CRC who had progressed up to 3 months after discontinuing first-line bevacizumab plus chemotherapy were assigned to receive second-line chemotherapy with or without bevacizumab. Continuation of bevacizumab led to a significant improvement in OS from 9.8 to 11.2 months ($p=0.0062$). Although the magnitude of benefit associated with continuing bevacizumab treatment beyond progression observed in this trial was much less than suggested in the ARIES and BRiTE studies, this trial provided strong evidence with respect to the point that stable resistance to bevacizumab had not developed. Interestingly, bevacizumab beyond progression has been associated with an overall survival benefit in a retrospective study of 23 patients with recurrent

glioblastoma[95], and single-agent bevacizumab has been shown to be effective in five patients with relapsed epithelial ovarian carcinoma who had received first-line maintenance bevacizumab[96]. This indicates that VEGF expression may continue to be important for tumour growth despite progression, potentially in several tumour types.

## Irinotecan in CRC

At the start of the last decade, new biological agents were being tested in novel combinations in patients with chemotherapy-refractory CRC. The randomized phase II BOND trial[97] had an interesting trial design that demonstrated the validity of treatment beyond progression, even if it was not designed with this outcome in mind. In this trial, 329 patients with metastatic CRC that had failed irinotecan-based therapy prior to enrolment were assigned to receive either cetuximab monotherapy or cetuximab plus irinotecan. Interestingly, compared to cetuximab monotherapy, treatment beyond progression with cetuximab and irinotecan led to significant improvements in ORR (22.9% versus 10.8%, $p=0.007$), TTP (4.1 months versus 1.5 months ($p<0.001$) and a trend toward improving overall survival (8.6 months vs. 6.9 months, $p=0.48$). This trial led to the FDA approval of cetuximab in 2004 for the treatment of metastatic CRC. It was suggested that cetuximab re-sensitized tumours to irinotecan[97], but an alternative explanation is that irinotecan resistance had not developed at initial disease progression.

## Other examples

Additional randomized data for continuing treatment beyond progression are unavailable; however, this area is being explored in situations where drug rechallenge has demonstrated efficacy. In a retrospective study of 64 patients with NSCLC, a significant improvement in overall survival was observed in patients who continued erlotinib therapy beyond disease progression compared to those who switched to chemotherapy (32.3 months versus 23.0 months, $p=0.005$)[98]. A single institution case control

study of patients with NSCLC treated with erlotinib showed that patients who continued erlotinib treatment beyond progression (*n*=25) had a longer overall survival from the start of progression compared to patients who discontinued erlotinib (*n*=16; 14.5 months versus 2.0 months, HR 0.154, p=0.0003)[99]. This benefit was not dependent on *EGFR* mutation status. The phase III IMPRESS trial will compare erlotinib plus chemotherapy treatment beyond progression versus chemotherapy alone after disease progression in NSCLC[100]. It has also been observed that patients with RCC who remain on sunitinib after disease progression experience prolonged disease control[101] or better survival than patients who discontinue the drug[102]. A detailed analysis of the sunitinib registration trial revealed that the growth rates of tumours in the vast majority of patients with RCC do not increase for hundreds of days on therapy, in contrast to the theory that growth accelerates after a period of response. Thus acquired resistance may not occur, and upfront intrinsic resistance to anti-angiogenic treatment is the type that may be applicable[103]. Where progressive disease is documented, continued sunitinib may be favourable compared to switching to other less effective antiangiogenic TKIs[103], but this remains to be assessed in clinical trials.

## RESISTANCE MECHANISMS

If heritable changes in the tumour dictate drug response in patients, how can use of the same therapy after disease progression sometimes be effective? There is limited data showing a lack of correlation between absolute drug resistance and mutations associated with drug resistance[67,69,104]. Of note, the presence of an alleged resistance-inducing mutation at progression does not imply causation of resistance[105]. A false assumption that the tumour was resistant at initial treatment and/or transient resistance mechanisms have developed may explain the apparent clinical benefits derived from drug rechallenge and treatment beyond progression strategies. Since little preclinical research has been

undertaken to replicate these clinical treatment strategies and define the mechanisms involved, a number of hypotheses have been proposed (Figure 4.4 and Table 4.1), some of which are discussed below.

## Disease progression, not necessarily resistance

*RECIST, progression on therapy and drug resistance*

In solid tumours, the current dominating paradigm for declaring resistance is the classification of progressive disease using the RECIST (Response Evaluation Criteria in Solid Tumours) criteria[106,107]. These criteria define progression as the growth of a tumour's unidimensional longest axis of at least 20% from baseline (or the sum of diameters of multiple target lesions) or the appearance of one or more new lesions. A response is defined as the opposite, i.e. tumour shrinkage, regardless of the time to that event. The RECIST criteria for progression based on tumour dimensions were defined through several iterations based on measurement precision, but not how this is associated with survival. These definitions create problems for interpreting clinical data. First, the failure to observe progressive disease may not imply drug sensitivity, but instead might indicate the tumour in a natural state of stability, regardless of treatment. Second, the baseline from which to evaluate tumour response is 'reset' when sequential treatments are evaluated. This means that a tumour growing at a constant rate regardless of therapy may take longer to achieve a 20% increase in size in subsequent lines of treatment. Third, it is certainly possible, even likely, that at the time of a 20% tumour growth some patients with extended times to progression are achieving clinical benefit through antitumour activity of attenuated tumour growth.

Response evaluation criteria may overestimate the effect of therapy—or lack thereof— while having little to do with the inherent drug sensitivity of a tumour, unless, perhaps, the progression on therapy is considered dramatic. On one hand the efficacy of drug rechallenge beyond progression in certain cases may be slightly exaggerated, it may also mean that discontinuing therapy in the case of a minor progression is ill-advised. Changes in morphological imaging of the longest diameter of a tumour

may be an inadequate assessment of the aggressiveness the tumour. Instead, volumetric or functional imaging might be better approaches, as has been shown for multiple types of cancer[108-112]. Such is also the case when tumour volume does not reflect the number of viable tumour cells, which can occur when a tumour is composed primarily of necrotic tissue.

*Relapse and drug resistance*

For tumours that respond to drug rechallenge after relapse, the simplest explanation is that the tumour cells were not resistant when therapy was discontinued. If treatment had continued indefinitely, progression on therapy would eventually develop in the absence of cure. Relapses that occur shortly after stopping a course of chemotherapy (tumour repopulation) require no inherent change in the chemosensitivity of cells[113]. Indeed even 'resistant' relapses in ovarian[13], CRC[15], and small-cell lung carcinomas[14] show some sensitivity to retreatment. The generalization that relapses are not resistant is a complex and controversial issue. Increasing the duration or intensity of chemotherapy does not always improve clinical outcome, and if it does the benefit is often marginal[114-116]. Additionally, undetectable, persistent tumour cells remaining following a complete response or adjuvant therapy cannot be eradicated by treatment (as functionally, they are resistant), but patients that subsequently relapse are often sensitive to drug rechallenge[12]. By contrast, if some relapses are originally resistant, these mechanisms of resistance may be transient, as discussed below.

*Partial resistance or drug synergism*

When disease progression occurs while a patient is on a combination of drugs, it is impossible to determine if resistance has developed to one, some, all (or none) of the agents. The effect of trastuzumab treatment beyond progression in breast cancer or bevacizumab in CRC may be explained by assuming that the cancer had developed resistance to cytotoxic chemotherapy only. Alternatively, if resistance had developed to the initial drug combination, the continued agent may retain some benefit if it combines

synergistically with a newly introduced agent. In preclinical models, trastuzumab demonstrated synergistic activity when combined with different chemotherapy agents[117,118], perhaps explaining its efficacy in multiple lines of therapy. This is difficult to observe in patients if the individual effects of each agent cannot be tested. Interestingly, such a trial has been inadvertently conducted in patients with CRC[119]. In the three-arm phase III EFC4584trial, 463 patients with metastatic CRC who had progressed on or soon after the combination of irinotecan, 5-FU and leucovorin (FOLFIRI), either continued treatment of 5-FU plus leucovorin alone, in combination with oxaliplatin, or received single agent oxaliplatin. The triplet chemotherapy combination provided the greatest survival benefit; for triplet chemotherapy TTP was 4.6 months, for doublet chemotherapy it was 2.7 months ($p<0.0001$ versus triplet chemotherapy), and for single-agent oxaliplatin TTP was 1.6 months ($p=0.03$ versus triplet chemotherapy)[119]. This trend was also seen for the overall response rate, which was 9,9%, 0% and 1.3%, for triplet, doublet, and single chemotherapy, respectively ($p<0.0001$ for triplet versus doublet chemotherapy)[119,120]. Thus, 'synergy beyond progression' may enable new efficacy for an agent to which a tumour had previously become resistant.

## Spontaneous reversal of resistance

In the absence of drug selection or other exogenous stimuli, clonal cells may spontaneously become heterogeneous in their intracellular signalling patterns, proliferation rates and drug sensitivity[121-124]. Even cancer stem cells, the minority subpopulation of cells proposed to persist during drug treatment, may not be stable[125-128]. For instance, tumourigenic cells derived from melanoma patients were found to be highly prevalent and tumourigenic independent of the expression of several putative stem cell markers, and moreover, had surface markers that were reversibly expressed within lineages of cells, without a hierarchically organization as proposed by the cancer stem cell model[125]. Cycling populations of drug-resistant and drug-sensitive stem-like cells can occur in the presence[126] or absence of drug selection *in vitro* and *in vivo*[127,128]. Sharma *et al*.[127] identified a small subpopulation of stem-like persister cells from

NSCLC cell lines that could survive a near-lethal treatment with erlotinib. Persister cells were found to be transiently tolerant to drugs through epigenetic chromatin modifications: cells spontaneously re-acquired drug-sensitivity in drug-free media, and similar drug-tolerant cells could arise *de novo* even in the absence of lethal treatment[127]. Thus, populations of tumour cells, putative stem cells or otherwise, may be dynamically drug-resistant and phenotypically unstable. Such dynamic tumour heterogeneity could serve as a survival tactic for a tumour in fluctuating environmental conditions, but this same instability could conceivably be exploited by drug rechallenge to enable additional tumour responses[127].

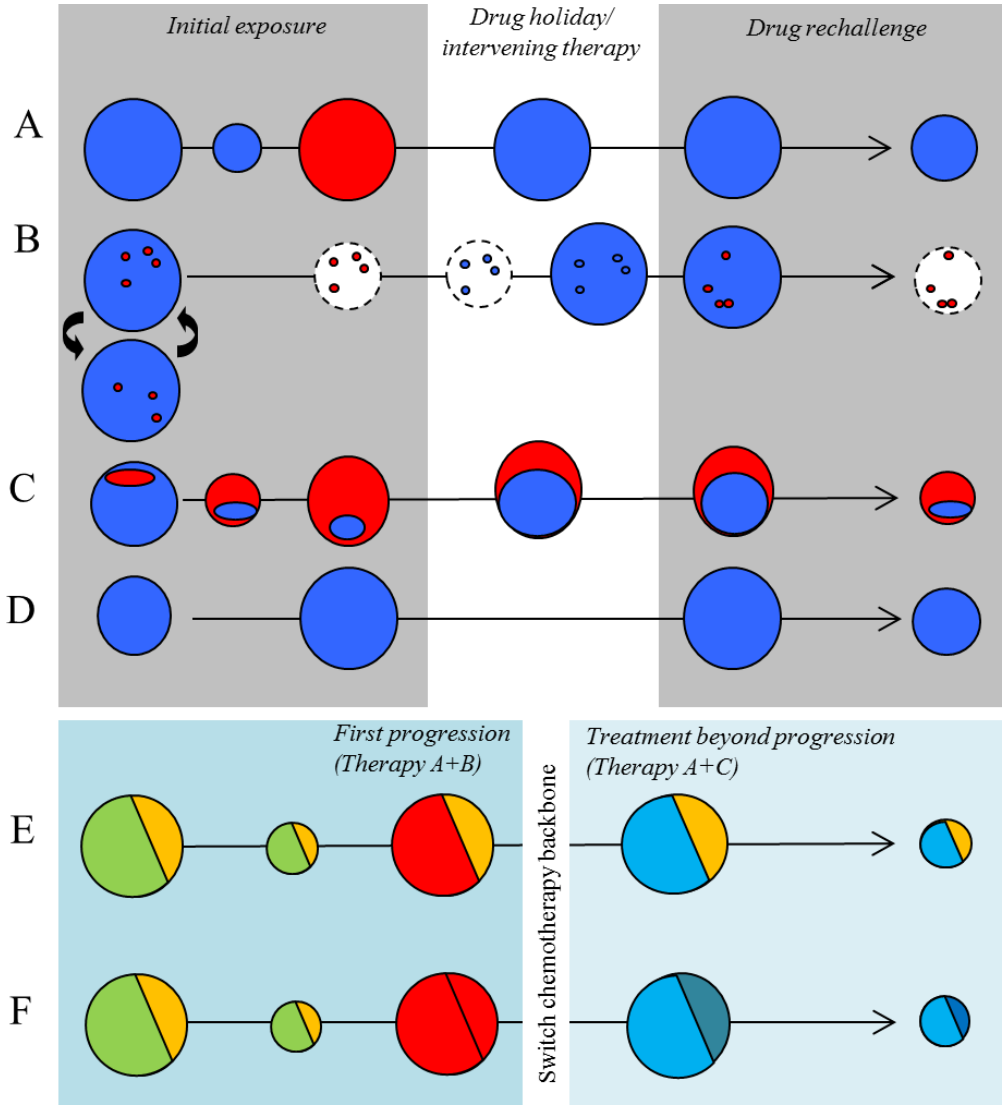


**Figure 4.4** Mechanisms of drug resistance during drug rechallenge (A-D) and treatment beyond progression regimens (E-F). **A**. Resistance caused by non-heritable cellular adaptation may be reversed by a drug holiday. **B**. Cells spontaneously cycle between drug-resistant and drug-sensitive states enabling cell survival at initial treatment and re-sensitization after therapy rechallenge. **C**. Altered proportions of fast-growing/sensitive and slow-growing/resistant cells results in tumour regrowth during a drug holiday or intervening therapy, but drug sensitivity at retreatment. **D**. A slow-growing tumour, that had not yet acquired resistance, may be mistakenly classified as progressive disease based on RECIST; therefore, it is sensitive to rechallenge therapy. **E**. At first progression the tumour had not yet acquired resistance to the agent used for treatment beyond progression; therefore, switching the chemotherapy backbone, but continuing the other agent may be beneficial. **F**. The tumour becomes resistant to both agents at disease progression. The agent continued beyond progression combines synergistically with a newly introduced agent, thus bypassing previous resistance mechanisms. Red denotes resistant cells or tumours.

## Drug holiday-mediated tumour re-sensitization

*Reversal of resistance mechanisms*

A drug holiday is a major reason cited for causing tumours to become re-sensitized to therapy. Indeed, loss of drug resistance is occasionally reported following drug withdrawal *in vitro*[127,129-133], although this is difficult to demonstrate *in vivo*. In xenograft models of RCC and hepatocellular carcinoma, tumours that had acquired resistance to sorafenib or sunitinib were shown to become re-sensitized to treatment after being transplanted into new hosts[134-136]. Long time periods are sometimes necessary to develop *in vivo* resistance, and the short doubling times of cancer cells in these hosts make drug holidays at disease progression ineffective interventions to improve therapeutic efficacy. Treatment interruption for 6 weeks was possible in mice that had developed resistance to the aromatase inhibitor letrozole in a model of hormone-dependent breast cancer[137]. Stopping therapy was found to restore tumour oestrogen receptor-α levels allowing effective rechallenge. This strategy has been adapted to the ongoing SOLE (Study of Letrozole Extension) phase III clinical trial[138].

In preclinical studies, treatment strategies using pre-planned pulsed regimens are often used. Although the planned drug holidays are not influenced by disease progression, this strategy has shown enhanced and prolonged antitumour activity compared to continuous administration in numerous animal models[139-141]. Melanoma tumour cells with acquired resistance to the BRAF inhibitor vemurafenib were found to depend on the presence of vemurafenib for continued proliferation, but, counterintuitively, suffered a growth disadvantage upon drug withdrawal leading to tumour regression[142]. Intermittent treatment was found to delay the onset of drug resistance *in vivo* as cells were sensitive to reintroduction of vemurafenib[9]. This finding suggests that adaptation to a drug-free environment may reverse or delay the onset of drug resistance and might explain resistance to drug rechallenge. This mechanism may also be relevant during treatment beyond progression when therapy is not given continuously[18,89,94,97]. For example, 30.8% of all patients who continued treatment beyond progression in the BRiTE study

discontinued bevacizumab for more than 28 days prior to its reintroduction[18]. In the TML study, therapy breaks of up to 3 months were allowed, even if they preceded initial disease progression[94].

**Table 4.1** Mechanisms of acquired resistance during drug rechallenge and continuation of treatment beyond progression

| Possible mechanism(s) | Drug rechallenge (progression off therapy/relapse) | Drug rechallenge (progression on therapy) | Continuation of treatment beyond progression (TBP) |
|---|---|---|---|
| Permanent/mutational resistance mechanism driving initial progression to agent | No | No (unless residual sensitive cells are fast-growing) | No (unless new combination acts synergistically by new mechanism) |
| Tumours are not resistant to agent at initial progression | Yes | Yes | Yes |
| Reversible resistance to a drug-free environment caused by tumour cell adaptation | Yes | Yes | Unlikely<br>(Yes if TBP discontinuous) |
| Tumour cells spontaneously cycle between resistant and sensitive states | Yes | Unlikely | Unlikely |
| Drugs combine synergistically after progression | No | No | Yes |

*Altered tumour kinetics*

Therapeutic intervention and subsequent drug withdrawal could favour or limit the growth of different populations of cells within a heterogeneous tumour in a way that could be manipulated by drug rechallenge. Initial treatment of a tumour consisting of fast-growing and slow-growing cells will mostly affect the fast-growing cells, inducing a clinical response. Eventually the slow-growing cells will cause tumour progression and, if therapy is discontinued, regrowth of fast-growing cells provides an

opportunity for the tumour to respond to retreatment. An intervening therapy, rather than a therapy break, might also allow regrowth of drug-sensitive cells[58,68,80]. A similar explanation has been proposed to explain sensitivity to EGFR TKI rechallenge in NSCLC, in which acquired resistance is often thought to be due to *EGFR* mutations, such as the T790M mutation. A loss of T790M during a TKI-free interval has been observed in patients resistant to EGFR TKIs who presented at disease progression with the mutation. Moreover, these patients were sensitive to rechallenge[143,144]. Thus TKI withdrawal may reduce the proportion of T790M mutant cells[132,143]. Even tumours consisting of a mixture of sensitive and resistant cells may behave more like sensitive tumours in response to treatment as the higher degree of growth inhibition and shorter doubling times of sensitive cells may mask therapeutic effects on resistant cells[145]. This suggests that even genetic resistance mechanisms may be reversible.

## DISCUSSION

### Summary of clinical findings

As discussed above and previously[12], drug rechallenge and treatment beyond progression strategies can be efficacious for a surprising number of patients with metastatic cancer. A number of interesting observations or implications emerge from these data. 1. Further clinical benefit can be achieved from reuse of the same drug after disease progression whether progression occurred on therapy[58,67,74,79] or after its discontinuation[26,35,40,44,55,146,147]. Based on the limited available data, continuation of treatment beyond progression may yield an overall survival benefit[91,94], whereas certain drug rechallenge regimens offer long-term benefits that are similar to continuous treatment[26,27,39,41]. This must be confirmed in randomized studies for additional malignancies and drug treatments to understand the true scale of these observations, and to assess how retreatment compares with changing to an alternate therapy[73]. 2. Rechallenge strategies seem to be broadly effective (Figure 4.2) and do not appear to

discriminate between the class of anticancer therapy used[27,34,57,67,79,148,149], the tumour type[13,23,37,44,55,67,74,79,150,151], the compartment targeted (the genetically unstable tumour versus the genetically stable stromal cells)[27,44,57,79,96] or the type of progression (on or off therapy) [44,79]. Treatment beyond progression has shown activity in a variety of tumour types with both targeted agents and chemotherapy (Figure 4.3)[90,91,94]. 3. Based on the high response rates achieved, rechallenge regimens do not appear to select resistant clones or accelerate acquired secondary resistance. This may be particularly true of disease that recurs after therapy discontinuation in which rechallenge tends to elicit the strongest responses, however there is limited data available comparing rechallenge responses following different types of progressions to confirm this[40,44,55,74]. While data are immature on treatment beyond progression, classic resistance mechanisms do not seem to apply here either[89,94,97]. 4. The length of the interval between rechallenge treatments, in relapse and progression on therapy settings, has been positively associated with the response to rechallenge (References [26,34,40,79,147,152] and Figure 4.2b), similar to what has been established with rechallenge of platinum therapy in patients with relapsed ovarian carcinoma[13]. It is tempting to think that a prolonged interval between exposures increases the magnitude of the 'reversal effect' on drug resistance. However, it should be noted that patients with a shorter intervening period might have more-aggressive disease such that any therapy may lead to weaker responses of shorter duration. 5. The degree of the initial objective response is also associated with a positive response to rechallenge[30,40,58,67,146,152] therefore highlighting the importance of primary/intrinsic resistance rather than acquired resistance in response to therapy. 6. PFS is almost always shorter and objective responses weaker at rechallenge (Figure 4.2a,b and references [20,34,40,58,67,79,153]), suggesting that resistant cells eventually dominate a tumour rendering treatment eventually less effective—or this simply underscores how subsequent lines of therapy are less effective compared to previous ones while the tumour becomes increasingly aggressive over time (Figure 4.2b). 7. Patients that tolerate a first course of therapy are likely to tolerate a second course of the same therapy. Toxicity is typically non-cumulative at rechallenge exposure[27,35,39,55,67,72,79,146,152], although neurotoxic drugs might be an exception[23].

## Implications for drug resistance

The data suggests that the definitions and implications of clinical drug resistance must be used and applied carefully. When early single-arm clinical trials tested P-glycoprotein antagonists in combination with cytotoxic chemotherapy—the same chemotherapy that tumours had previously become resistant to—the high response rates that were observed were interpreted as evidence of a reversal of multidrug resistance, sparking hundreds of millions of dollars of further research and development[153,154]. Subsequent randomized phase III trials demonstrated no benefit of these antagonists[7,155,156], suggesting that the earlier observed efficacy may have been due to unintentional drug rechallenge. While most oncologists would likely agree that rapid tumour progression during a course of therapy signifies drug resistance, the reasons for minor progressions after sustained therapy or progression off therapy are more controversial. Based on the available evidence, early discontinuation of therapy (for reasons other than progressive disease) does not appear to select for drug-resistant clones at relapse. If therapy had been continued until progression one cannot, however, be certain that a tumour is truly and permanently drug resistant unless it is shown to no longer be responsive to the same therapy. It is clear that RECIST-defined progression does not denote a meaningful selection of drug-resistant clones nor is it a gold standard indicator for when to change a course of therapy. We suggest that these concepts, along with the notion of reversible or unstable drug resistance, should be given greater emphasis in oncological research and in clinical practice.

## Implications for clinical practice

The main implication of transient or reversible drug resistance is that an old agent should not be uniformly excluded from further use in a patient if previously found to be effective and well-tolerated. This possibility has many potential benefits. First, if a patient on long-term therapy is suffering from adverse events or desires a drug holiday, early interruption of therapy might be considered without the

concern of accelerating drug resistance, worsening toxicity and potentially compromising overall survival. With a high likelihood of response at re-exposure, further quality-of-life benefits are possible through reduced number of overall treatments and hospital visits. Second, the option of repeating or continuing a therapy considerably improves the availability of therapeutic options. If all other treatment options have been exhausted or there is no standard of care, continuing the same therapy indefinitely or drug rechallenge may be viable options. This may also be the case if other agents are available. Consider a new agent with unclear real-world efficacy and a new toxicity profile. This new agent may offer only a marginal benefit in PFS or overall survival with potential reductions in quality of life. Third, if a previously used drug is off-patent and inexpensive, and/or the new agent is expensive, a cost-benefit assessment may favour retreatment. Strategies to make cancer care more affordable and accessible are in high demand given the rapidly rising cost of many new cancer drugs and patient care[157,158]. Continuing a high-cost drug, such as bevacizumab or trastuzumab, beyond progression can be troubling to some given the modest improvements in survival that these agents provide. Finally, if continuing therapy after progression incrementally improves patient outcome there are clear implications for clinical trial design. In a trial of a novel agent or strategy seeking an overall survival benefit, consideration should be given to allowing therapy continuation through a minor (to be prospectively defined) progression in the absence of demonstrated benefit of alternate therapies.

## Limitations

Is it possible that retreatment or continuation of treatment could be active in all tumour types and with all drugs? There are clinical examples in which non-heritable resistance mechanisms may have a role, for example, when tumours display a lack of cross-resistance to agents within the same drug class e.g. different taxane and platinum agents in breast cancer and ovarian cancer treatments, respectively[13,150] or antiangiogenic TKIs in RCC[77]. The overall success of these strategies across a broad spectrum of malignancies and anticancer agents—and that we were unable to find any evidence of absolute

inactivity—suggests that unstable and non-heritable resistance mechanisms have a significant role in medical oncology. Of note, much of the present literature consists of anecdotal studies clearly subject to various biases. The selection bias of retrospective or single-arm trials—patients selected for retreatment often have good performance status and prognosis, and experience good initial responses to treatment—may cause an overestimation of the activity of the treatment strategy. Furthermore, publication bias may further skew this apparent efficacy.

## FUTURE DIRECTIONS AND CONCLUSIONS

While a few phase III clinical trials demonstrate that continuous conventional maximum tolerated dose (MTD) chemotherapy offers no survival benefit and poorer quality of life compared to rechallenge-like regimens[13,22,159,160], one can only speculate on the true efficacy of drug rechallenge using most of the newer therapies. Further research is needed to address this area. If research effort focuses only on identifying and targeting robust and stable changes that occur in the tumour cell, more subtle explanations for resistance will be missed. Preclinical studies of drug rechallenge and treatment beyond progression, which are rare, are necessary. Such studies require that questionably relevant experimental models of cancer, such as in vitro dose escalation and selection of clones, are used with caution and that models reflecting the clinical situation, such as models of advanced metastatic disease[161], genetically engineered mouse models or patient-derived tumour xenografts[162] treated with relevant drugs and regimens, are implemented.

Future clinical studies are required to assess the impact of RECIST-determined progression with subsequent survival and to consider whether alternative growth percentages, time-based rates of change, or newer measures are more predictive of long-term outcomes. Well-designed prospective phase III

clinical trials with appropriate controls are necessary to compare if switching to a new therapy (conventional therapy) is better than a retreatment strategy. Based on this Review, and as suggested for trials in RCC[103] an appropriate control arm for testing a new therapy may be continuing or repeating an old therapy. Unfortunately, it may be difficult to accrue patients into a clinical trial if therapy is discontinued temporarily or a new agent is not offered. A number of questions should be addressed in future studies. When 'resistance' develops to an initially effective long-term continuous low-dose metronomic chemotherapy, would a break in therapy at disease progression similarly reverse the resistant phenotype? How do therapy dose (MTD versus low-dose chemotherapy) and regimen (dose dense versus continuous and protracted/metronomic dosing) contribute to delaying or preventing the onset of stable acquired resistance[163]? When is some sort of maintenance therapy, during what would otherwise be a therapy break, be superior to a true drug holiday in terms of re-sensitizing a tumour to therapy[24,164,165]? Would continuing all agents beyond progression be of equal benefit to switching the chemotherapy backbone? Is continuous (beyond progression) treatment superior to a treat-as-needed approach?

In conclusion, there are abundant clinical examples in medical oncology of transient resistance or progression that do not equate to resistance. Despite the development of disease progression on or after discontinuing therapy, a large number of patients remain sensitive to therapy that is continued or reintroduced at a later time. Such treatment strategies have major implications for patient care, the choice and timing of therapies, and the overall quality and cost of treatment. These considerations simply serve to highlight the shortcomings of present concepts of drug resistance and how progressive disease is characterized and managed.

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# Chapter 5. Discussion and Conclusions

## DISCUSSION

The results of my studies have added a new dimension to a critically important area of tumour angiogenesis and anti-angiogenic therapy research: the basis of acquired resistance to anti-angiogenic drugs – in this case an oral small molecular TKI, sorafenib, approved for treatment of HCC and RCC patients. Moreover my results help explain the basis of the reversible or unstable resistant phenotype to sorafenib, and by extension other forms of such reversible resistance to other antiangiogenic TKIs including sunitinib.

### MECHANISMS OF REVERSIBLE SORAFENIB RESISTANCE IN THE HEP3B-HCG MODEL

As our laboratory previously observed, sorafenib resistance in immune-deficient mice bearing orthotopic HCC tumours is a reversible phenotype[1]. The mechanisms of sorafenib resistance in the Hep3B-hCG tumour model were therefore investigated in detail. The following explanations for resistance were uncovered: 1) a gradual decline in systemic drug exposure levels over time and 2) a switch to and increased reliance on vascular co-option during treatment which was facilitated by increases in cancer cell invasion. Both mechanisms may explain the aforementioned observation of loss of resistance, which occurred by dissociating cancer cells from drug-resistant tumours and re-implanting them into new hosts after a period of expansion *in vitro*[1]. Removal of the cancer cells from the liver microenvironment and prolonged drug withdrawal could disrupt the tumour-stroma interaction that is critical for vessel co-option, reverse the effect of a large tumour on drug metabolism-mediated clearance and reverse several changes in gene expression that were affected by treatment.

Independently, each of the aforementioned resistance mechanisms can explain the slow progression and acquired resistance of HCC tumours during sorafenib treatment. A switch from perfusion mediated by angiogenesis to perfusion as a result of co-option provides a clear explanation for why liver

tumours are able to grow during concurrent (and near-complete) angiogenesis inhibition. Additionally, a gradual reduction in drug exposure levels over time may reduce the potency of target inhibition and consequently the anti-tumour effect. What is unclear is how, if at all, these mechanisms are related and to what degree each is important for the observed pattern of acquired resistance. For instance, are they co-dependent mechanisms? Do they co-operate additively to produce resistance? Do they act independently such that in the absence of the other, resistance still develops?

One reason these questions are raised is that in the original Hep3B-hCG sorafenib resistance study a tumour growth pattern of initial anti-tumour response followed by tumour progression occurred in *subcutaneously* implanted tumours on approximately the same time scale as intrahepatic tumours[1]. Without the highly perfused liver vasculature, it is unlikely that vessel co-option could occur under the skin (though co-option has been observed in human primary melanomas[2]). In such cases could drug exposure levels therefore be promoting resistance on therapy?

Another reason to question the involvement of both mechanisms is based on the fact that the most apparent anti-tumour mechanism of action of sorafenib in Hep3B-hCG tumours was anti-angiogenesis. If one were to assume that VEGFRs (the kinases considered most important for angiogenesis) are what drive HCC growth, one would expect a dose-response effect such that when drug levels adaptively decline the anti-angiogenic effect would be diminished. This was not the case and the direct consequence of low drug exposure levels on the tumour itself was not determined (though the effect of sorafenib treatment on VEGFR2 phosphorylation also could not be confirmed in my studies, Chapter 2)[3]. It seems likely that other off-target and/or direct anti-tumour effects of the drug could be important for mediating some of the effects of sorafenib treatment. Indeed this is an ongoing debate for sorafenib and other antiangiogenic agents[4-6] and some investigators have suggested that these drugs have direct pro-apoptotic activity[7,8]. For instance, only sorafenib has shown superior efficacy in HCC over some of the more potent and specific inhibitors of VEGF receptors such as linifanib and ramucirumab, respectively[9]. Whether for

biological or technical reasons, inhibition of VEGFR or PDGFR phosphorylation could not be linked to the effect of sorafenib, the issue of the mechanism of activity of sorafenib remained unresolved. As mentioned in Chapter 2, drug level decline could play a minor role in drug resistance. Without fully understanding the anti-tumour mechanisms of sorafenib it is difficult to tease out these complexities.

## OTHER PROPOSED MECHANISMS OF RESISTANCE TO SORAFENIB IN HCC

The resistance mechanisms uncovered from my studies contrast with the other published reports on sorafenib resistance. Numerous possible mechanisms have been proposed which provide alternate explanations for the upfront or acquired loss of response to sorafenib in HCC. Each new proposed mechanism adds new possible solutions to the problem but also causes confusion regarding the identification of the most promising new therapeutic target. The major proposed resistance mechanisms are outlined and discussed below.

### 1. Altered metabolism of HCC cells

Shen et al. screened various HCC cell lines *in vitro* and observed a strong correlation between sorafenib IC50s and enhanced glycolysis by blocking oxidative phosphorylation with an ATP synthase inhibitor oligomycin[10]. Enhanced glycolysis was also observed upfront in Hep3B cells and in *in vitro*-generated resistant Huh7 cells (derived from long-term sorafenib treatment of cells). Dichloroacetate (DCA), a pyruvate dehydrogenase kinase inhibitor, reversed sorafenib resistance, reduced glucose uptake and activated oxidative phosphorylation *in vitro*[10]. Combination treatment of DCA with sorafenib also enhanced tumour growth inhibition and apoptosis in a subcutaneous Hep3B tumour model relative to DCA (100 mg/kg/day) or sorafenib (10 mg/kg/day) treatment alone. These data suggest that reversing the Warburg effect could drive HCC cells to undergo apoptosis during sorafenib treatment.

**2. Induction of pro-survival and proliferative pathways**

Chen et al. also selected for sorafenib resistant cell lines from Huh7 cells (Huh7-R1 and Huh7R-2) by slowly ramping-up the dose of sorafenib *in vitro*[11]. An induction of the PI3K/AKT signalling pathway (induction of P-Akt and AKT and PTEN down-regulation), which is important for cell survival and proliferation, was observed. An AKT inhibitor sensitized Huh7-R1 and Huh7R2 cells to sorafenib-induced apoptosis *in vitro*. Potentially, PI3K/AKT signalling could be a compensatory effect of sorafenib treatment as high cross-talk between this and the Raf-ERK pathway is known to occur[12].

By delivering a library of transposon-mediated small hairpin RNAs targeting oncogenic driver genes in an inducible mouse model of HCC, an alternate cross-talk pathway mediated by Mapk14 (p38-α) was found to be aberrantly expressed during sorafenib treatment[13]. Further *in vitro* and *in vivo* studies found that induced Mapk14 expression could promote HCC cellular and tumour growth during Raf/ERK/MEK blockade and Mapk14 pathway inhibition could potentiate the anti-tumour effects of sorafenib.

Sorafenib has been shown to activate cellular autophagy, a cytoprotective or death-promoting (depending on the context) self-digestion process induced under starvation conditions, in which cellular proteins and organelles are sequestered in autophagosomes and degraded by fusion with lysosomes[14]. HCC cell lines treated with sorafenib were found to promote accumulation of autophagosomes demonstrated by the accumulation of LC3-II autophagosomal marker *in vitro*[15]. Genetic or pharmacologic inhibition of autophagy by ATG7 siRNA or chloroquine enhanced the apoptotic effect of sorafenib in cells and potentiated the anti-tumour effect of sorafenib in subcutaneous Huh7 xenografts[15]. Thus, autophagy was proposed as a possible mediator of resistance to sorafenib in HCC, which could be targeted to increase the therapeutic effect of sorafenib.

Two studies have also implicated EGFR activation during upfront sorafenib resistance[16,17]. EGFR activity correlated with sorafenib IC50s for clonogenic survival, with Hep3B cells being the most resistant and EGFR-activated and Huh7 or HepG2 cells the least so[16]. Treatment with an EGFR TKI (gefitinib or erlotinib) or antibody (cetuximab) sensitized resistant cells to sorafenib treatment whereas the EGFR ligand amphiregulin promoted clonogenic cell survival. An additional study corroborated this enhanced anti-tumour effect of sorafenib plus EGFR inhibition in HCC cells and in a subcutaneous xenograft model using the PLC cell line[17]. The authors noted significant up-regulation of amphiregulin in sorafenib-treated HCC patients, however the importance of EGFR signalling during sorafenib resistance has been called to question by the absence of overall survival benefit by combining erlotinib with sorafenib relative to sorafenib plus placebo in a phase III clinical trial of first-line advanced HCC[18].

**3. Cancer stem cells**

Xin et al. reported that Cyanine-5-dUTP label-retaining cancer cells (LRCC) are a subpopulation of cells isolated from HCC cell lines representing cancer stem cells with pluripotent gene expression, asymmetric cell division and tumour initiating properties[19]. The authors observed that LRCC were resistant to sorafenib with reduced apoptosis, improved viability and enhanced AKT and ERK signalling compared to non-LRCC. Sorafenib-treated LRCC upregulated cancer stem cell marker ALDH1, genes involved in cell survival and Wnt signalling, and down-regulated cell adhesion and apoptosis genes. LRCC were proposed to play a role in HCC recurrence during sorafenib therapy[19] but this was not tested *in vivo*. The existence of stem cells from established and genetically homogenous cell lines is debatable. But most importantly, since sorafenib generally causes tumour stasis ('stable disease') rather than regression in responding patients (response rates on sorafenib are only 2-3%)[20,21], small populations of drug-resistant stem cells are unlikely to produce a substantial effect on the bulk tumour.

**4. Induction of EMT**

Van Malenstein and colleagues generated sorafenib-resistant cell lines from HepG2 and Huh7 HCC cells *in vitro* by gradually increasing the concentration of sorafenib in 0.25μM steps over the course of several months[22]. Resistant cells were found to gain properties of EMT including gain of spindle-cell morphology, increased cell invasion and changes in multiple EMT genes such as loss of E-cadherin and increase in vimentin expression. Resistant cells had increased ERK and AKT phosphorylation and were sensitized to sorafenib by an AKT inhibitor. Drug withdrawal in the cancer cells resulted in accelerated cell proliferation beyond the rate of untreated parental cells and reversal of some but not all of the EMT properties[22].

Another study observed that increased expression of the small heat shock protein αB-Crystallin in HCC patients was associated with vascular invasion, absent tumour encapsulation, poor survival and postoperative recurrence[23]. αB-Crystallin induced EMT through activation of an ERK1/2-Fra-1/slug pathway which limited the efficacy of sorafenib *in vitro*[23]. αB-Crystallin was found to increase HCC cell invasion *in vitro* and promote metastasis in orthotopic HCC models by inducing or blocking its expression.

**5. Tumour hypoxia**

Finally, Liang et al. analysed human HCCs that received sorafenib prior to liver surgery[24]. Significantly greater hypoxia and HIF-1α expression as well as reduced CD31 vessel density were observed in patients that progressed on sorafenib treatment vs. those that responded to treatment. Levels of HIF-dependent genes (CXCR4, GLUT-1, MDR1, VEGF and CA9) were also induced in resistant vs. sensitive and control human tumours. Treatment of various non-resistant/parental HCC cells with sorafenib under hypoxia (1% $O_2$) *increased* cell viability which was reduced using a HIF-1α inhibitor (EF24) or lentiviral shRNA. Thus hypoxia provided HCC cells with a growth advantage during sorafenib treatment. Notably, EF24 in combination with sorafenib led to synergistic reductions of orthotopic and

subcutaneous Huh7 tumour growth and development of metastatic foci following intravenous injection of HepG2 cells[24]. Unfortunately *in vivo* combination treatments were tested in sorafenib-sensitive tumour models, which based on their patient data, would not be associated with hypoxia. Thus, the effects of co-targeting hypoxia and sorafenib during resistance could not be properly assessed.

## Summary of resistance mechanisms

With only two exceptions[13,24] there is a notable lack of studies investigating sorafenib resistance mechanisms operating *in vivo.* This is critical since the primary proposed mechanism of sorafenib in targeting the tumour stroma (anti-angiogenesis) is disregarded. Moreover the acquired resistance models described above[10,11,23] are exclusively derived from prolonged *in vitro* drug exposure to escalating and supra-physiological concentrations of sorafenib – situations unlikely to occur clinically. There is therefore the danger of producing artefacts from such cell culture studies. This does not necessarily invalidate the findings, however the results should be considered in context of tumour heterogeneity and the tumour microenvironment. Perhaps these *in vitro* studies unveil the subtle direct-tumour targeting effects of the drug which are otherwise masked by the dominant anti-angiogenic/anti-stromal effects of the drug *in vivo.*

Superficially these proposed mechanisms appear distinct but in many cases they may be interrelated via converging up- or downstream signalling events. For example, cells that have undergone an EMT and stem-like cells share properties including expression of stem cell markers, signalling pathways (e.g. Wnt, Notch, TGF-β), dedifferentiation from epithelial cells, the ability to form new tumours or metastases and drug resistance, and stem-like cells can form through an EMT[14]. Activation of the AKT signalling pathway may promote EMT[25] and could be a driver of autophagy in sorafenib-resistant cells *in vitro*[26]. A possible working model is that anti-angiogenic treatment results in increased hypoxia leading to activation of HIF-targeted genes and promotion of adaptive survival cell processes

including EMT, maintenance of a stem-like state or autophagy[26,27]. The linkages between these mechanisms need to be further evaluated.

## Vessel co-option and acquired resistance in HCC

The mechanisms proposed above are not inconsistent with vessel co-option. Although the vascular effects of sorafenib in HCC have not been well characterized, it is notable that compensatory 'rebound angiogenesis'[28] has not yet been brought forward as a contributor to resistance in HCC. In agreement with the above studies, EMT and hypoxia could be important drivers of cancer cell invasion and subsequent co-option of the vasculature. Adaptive survival pathways such as autophagy could take place in parallel to promote cancer cell survival and resistance to apoptosis during a shut-down of the vascular supply.

There is very limited clinical or preclinical evidence of involvement of vessel co-option in resistance (either acquired or intrinsic) to an angiogenesis inhibitor. We are however aware of another study by the group of Dr. Andrew Reynolds in the UK (personal communication, manuscript in review) that has reached similar conclusions as us, regarding colorectal liver metastases in the context of anti-angiogenic treatment. The histological growth patterns of human colorectal liver metastases from patients treated pre-operatively with bevacizumab and chemotherapy were analysed. Liver metastases with a 'replacement' growth pattern - in which tumour cells invade into and interdigitate with the sinusoids of the liver parenchyma – were found to reflect tumours utilizing vessel co-option at the liver-tumour interface, in contrast to metastases with desmoplastic or pushing growth patterns – both of which characterize well-circumscribed and angiogenic tumours. Importantly, lesions with a predominantly 'replacement' growth pattern were associated with poor pathological response and the incidence of such corresponded to both intrinsic resistance *and* acquired resistance – ie. replacement growth pattern could be observed prior to and following the course of bevacizumab treatment, or it developed only following

treatment, respectively. In conjunction with my findings, these data provide strong support of the role of vessel co-option during resistance not only preclinically, but also in patients.

A final factor to be considered regarding clinical drug resistance mechanisms is that of intratumoral heterogeneity. Recent sequencing and chromosome analyses on multiple spatially separated tumour samples from RCC tumours revealed extensive variability of somatic mutations and chromosomal aberrations within the same tumour29. Similar observations were made in HCCs30,31. Such heterogeneity may cause region-specific dominant tumour cell growth-promoting pathways, alterations in the tumour microenvironment and unique resistance mechanisms. Conceivably, not only could a subset of HCC patients have tumours which utilize vessel co-option during sorafenib treatment, but specific regions within a single tumour may utilize vessel co-option while other regions employ strategies such as autophagy or rebound angiogenesis.

## TREATING SORAFENIB-RESISTANT HCC

As discussed earlier, sorafenib remains the only approved drug for systemic HCC treatment, but since its efficacy is limited with high rates of adverse events, dose reductions and therapy discontinuation, new therapeutic approaches and treatments are in dire need. Angiogenesis inhibitors do not always clearly fit with the paradigm of permanent (stable/heritable) acquired drug resistance[1,32-37], therefore several therapeutic options which re-use the same drug may be applicable. Drug rechallenge after disease progression may be an option in situations and malignancies where an alternate intervening therapy is available or therapy can be stopped for long periods because tumour burden is minimal and the risk of relapse is low (ie. post-surgical resection), but such is not the case for advanced HCC. Three promising

potential strategies for HCC treatment are discussed below: treatment beyond progression, dose and schedule modifications and targeting vessel co-option.

## Sorafenib treatment beyond progression

Our data demonstrates that sorafenib therapy potently inhibited angiogenesis and this activity persisted over time, without any re-induction of angiogenesis mediated, for instance, by induction of alternative pro-angiogenic factors[28]. Maintaining anti-angiogenic suppression was beneficial long-term, even while tumours eventually progressed on-treatment because stopping therapy caused rapid angiogenic induction and accelerated regrowth (the latter being a well-described phenomenon[38-40]). Based on these findings, I propose that sorafenib should not necessarily be discontinued as a result of tumour progression. Likewise, a recent non-randomized prospective study in HCC patients indicated that discontinuing sorafenib after radiological disease progression increased the tumour growth rate compared to continuing on sorafenib treatment[41]. Moreover, continuing sorafenib beyond disease progression led to a significant overall survival benefit (n=36, 5.2 vs. 11.9 months, p=0.012). This strategy should be further investigated in prospective randomized trials.

Another consequence of the observed robust anti-angiogenic activity of sorafenib is that switching to a different angiogenesis inhibitor could be of little use relative to continuing sorafenib treatment. I performed additional therapy studies which assessed the activity of anti-angiogenic drug therapy switching in sorafenib-resistant Hep3B-hCG tumour-bearing mice (ie. to test possible second-line therapies). As shown in Figure 5.1, switching from sorafenib to another anti-angiogenic agent was not an effective strategy – alternate anti-angiogenic agents yielded poorer anti-tumour activity (e.g. pazopanib, DC101 – an anti-VEGFR2 monoclonal antibody or G6.31 – an anti-VEGF monoclonal antibody) or resulted in excessive toxicity (e.g. regorafenib - a more potent Raf/VEGFR2-targeted TKI than sorafenib with activity against additional kinases such as Tie2) compared to continuation of sorafenib treatment.

Regardless of the therapy, sorafenib as 'second-line' therapy was as good as or superior to the other treatments in terms of survival benefit. These data appear to recapitulate the failure of alternate anti-angiogenic therapies in HCC, particularly sunitinib and linifanib in first line HCC vs. sorafenib[42,43]. A phase III clinical trial evaluating regorafenib is currently underway in first-line and second-line (post-progression on sorafenib) HCC[44]. Currently the only comparator arm evaluated in this and other second-line therapy trials is placebo or best supportive care (BSC), but it could be argued that second-line sorafenib would be more appropriate.

## Sorafenib dose and schedule individualization

In addition to continuing sorafenib treatment long-term, dose individualization may be an important factor in the optimization of therapy. There are a few reasons why sorafenib-treated patients may require a dose other than the standard (ie. 400mg twice daily). First, high variability in drug exposure levels exist across patients given the same dose of sorafenib[45], and exposure levels can change (decline) over time on the same dose[45,46]. Maintaining adequate plasma drug levels would therefore require dose adjustments. Second, the tolerability of standard dose sorafenib is also highly variable. Many patients do not tolerate standard doses and would therefore require down-dosing, a drug holiday or permanent therapy discontinuation. The rate of discontinuation of treatment due to adverse events (without a prior dose-reduction) has been reported to be as high as 33% in field practice[47]. Other patients experience very few if any adverse events, and while the patient may be relatively happy with this outcome, the consequence may be that their plasma drug levels are sub-optimal and they are less likely to experience clinical benefit from treatment[48-51]. A similar consequence may come of patients whose adverse events tend to diminish over time[45,52,53].

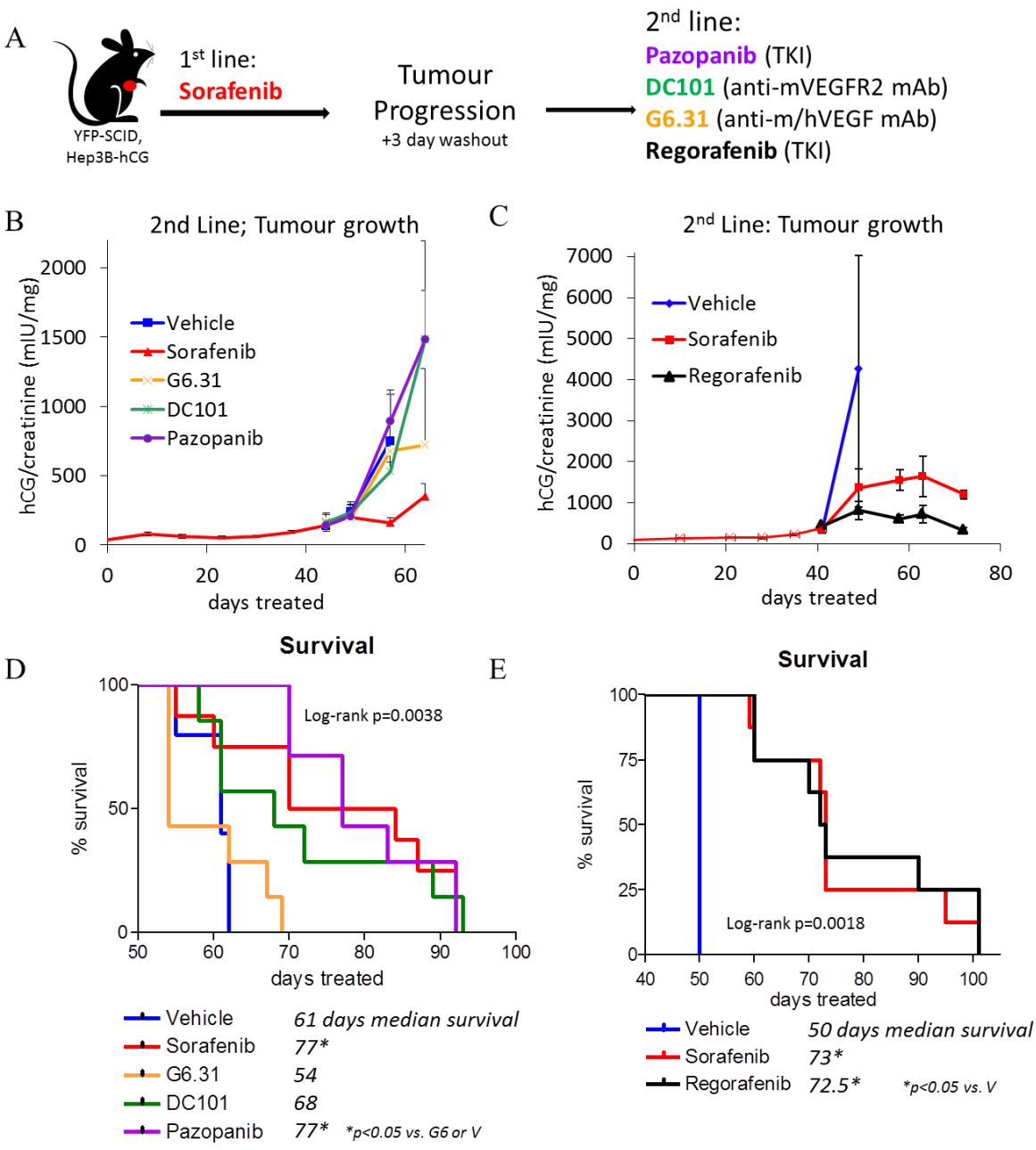


**Figure 5.1** The efficacy of switching to an alternate anti-angiogenic therapy in HCC. **A**. Schema of treatments of first line therapy (sorafenib) and second-line, alternate therapies. Monoclonal antibodies against VEGFR2 (DC101), VEGF (G6.31), and tyrosine kinase inhibitors pazopanib and regorafenib were tested in sorafenib-resistant Hep3B-hCG SCID mice. **B**. Sorafenib was the most effective second-line treatment in terms of tumour growth inhibition relative to G6.31, DC101 (800 µg/mouse) and pazopanib (150 mg/kg/day), but **C**. not relative to regorafenib (30mg/kg/day) which potently delayed tumour growth. **D-E**. Kaplan-Meier survival analysis demonstrates that sorafenib treatment as a second-line therapy was as effective, in the case of pazopanib or regorafenib, or superior to other tested agents, despite prior exposure of mice with this therapy and the development of acquired resistance.

As I also observed by dose-escalating sorafenib in mice, a higher dose generally has superior anti-tumour activity than a lower one, but the challenge with this is the increased toxicity that it brings. Dose reductions are necessary at times. In a retrospective field practice study[47], patients who reduced to half the dose of sorafenib for >70% of the treatment period were observed to experience significantly prolonged overall survival (21.6 months; 77 patients) compared to the patients (219) who remained on full-dose sorafenib or had dose reduced for <70% of the treatment period (9.6 months; p=0.0006)[47]. Treatment exposure time was 6.8 month vs. 3 months in the down-dosed and full-dose groups, respectively. This observation suggests that dose reduction may not necessarily reduce the therapeutic benefit of the drug, provided that the therapy is continued long-term[47,54].

The only completed prospective clinical trial to evaluate dose escalation in HCC was a phase II trial in which standard-dosed patients who experienced radiologic disease progression were escalated from 400mg to 600mg twice daily or given BSC. This trial was considered negative since the primary endpoint (PFS) was not met (PFS sorafenib 3.91 months vs. BSC 2.69 months, p=0.086)[55]. It is quite possible that a more efficacious treatment protocol could be assessed. A more optimal trial design might incorporate smaller dose escalation increments to improve tolerability, earlier and more frequent dose escalations if toxicity is manageable, fractionated dosing (ie. 3-4 times daily dosing) to improve drug absorption at higher doses[56] and regular allowance of down-dosing or drug holidays until unmanageable toxicity is reversed. Thus the goal would be to maintain patients on sorafenib therapy for as long as possible while on-treatment adverse effects are kept at moderate but acceptable levels. Further prospective evaluation of dose and schedule modification strategies is warranted.

## Targeting vessel co-option

My data suggests that some molecular changes associated with resistance may be reversed by schedule modifications (ie. breaks in therapy), but this strategy is not likely to prevent a tumour's

tendency to co-opt the surrounding liver vessels. Without targeting the predominating resistance mechanism, the dose and schedule modifications may only lead only to minor incremental changes in progression-free or overall survival. The notion of blocking the complete vascular supply of a tumour – that is, angiogenic and co-opted vessels – may therefore be a more impactful strategy. There are two primary ways that vessel co-option might be therapeutically targeted, particularly in the context of angiogenic suppression: 1. through inhibition of the cellular invasion process that precedes and accompanies co-option; and 2. by blocking the co-opted vasculature directly.

Inhibiting cancer cell invasion may serve as a method to prevent recruitment ('hijacking') of the host tissue blood vessels. To this effect, some potential therapeutic agents include c-Met/HGF antagonists57, EMT signaling inhibitors (e.g. TGF-β receptor kinase inhibitor58) and matrix metalloproteinase (MMP) inhibitors59. The latter is an interesting prospect since phase III clinical trials undertaken 20 years ago evaluating anti-invasive MMP inhibitors in combination with chemotherapy in non-small cell lung cancer were negative60. However, combining such agents with anti-angiogenic therapy instead might be a more promising approach. Chemotherapy is an alternative potential anti-co-option agent. Chemotherapy was recently reported by our laboratory to block the effect of anti-VEGFR2 antibody treatment on increasing local tumour invasion in vivo61. This finding may explain the absence of pro-metastatic effects observed in clinical studies of patients treated with anti-VEGF therapy concurrently with chemotherapy61 in contrast to preclinical studies of anti-angiogenic monotherapy in mice causing increases in local invasion and/or metastasis62,63. Chemotherapy combinations in HCC in particular are not likely to be a feasible approach, but could be investigated in other malignancies.

Directly blocking co-opted host vessels is a second appealing approach. Unfortunately there is the real risk that such vessels are too similar to their normal tissue counterparts such that any vascular targeted therapy would not have a reasonable therapeutic ratio. Molecular markers of co-opted tumour vessels which are distinct from the tissues of origin are presently unknown. If paracrine effects of the

surrounding cancer cells or separation of co-opted vessels from the normal tissue architecture somehow alter the molecular phenotype of co-opted vessels, there might be opportunity to exploit these differences. For instance, PD-L1, the ligand for PD-1 and a negative regulator of effector immune responses64, can be induced during inflammation on epithelial tissues, cancer cells and endothelial cells including liver sinusoidal endothelial cells65,66. PD-L1 has been shown to be induced during anti-angiogenic therapy treatment including sorafenib in models of HCC67,68. Additionally, anti-angiogenic therapy is showing promising synergy with immuno-therapy in preclinical models and also in some early clinical trials67,69-71. It is feasible that pro-inflammatory cytokines (IFN-γ, TNF-α) and growth factors from the neighbouring tumour stroma or cancer cells72 could induce PD-L1 on the adjacent co-opted endothelium, thus priming cells for anti-PD-L1-targeted immunotherapy and immune destruction. Ongoing clinical trials are exploring the combinatorial approach of anti-VEGF plus immunotherapy64.

## LIMITATIONS OF THE EXPERIMENTS

The key limitation of the presented work on HCC is that the Hep3B-hCG mouse model used may not accurately reflect human HCC disease. Importantly, liver cancer cells of a single cellular origin were injected into healthy, non-cirrhotic livers of immune-compromised mice. Orthotopic implantation of HCC cells has the advantage of recapitulating the site of growth of natural HCCs and displays histological growth patterns very similar to the human disease counterpart. Only by using this methodology could the mechanism of co-option during resistance be identified. However HCC is a highly heterogeneous disease. Use of one cell line derived from a single clone may reflect a single tumour 'type'. For instance, the Hep3B cell line is a hepatitis B virus-integrated HCC derived from one patient, which represents one of the most common risk factors and inducers of HCC.

HCC is a disease of double pathology including both liver disease (cirrhosis) and cancer and the underlying liver disease may have important consequences for therapy and its mechanisms of escape. In cases of viral (hepatitis B and C) and alcohol-induced liver damage and HCC development, liver tissue is commonly damaged by oxidative stress and inflammation, which causes a cycle of necrosis and liver regeneration[73]. This promotes hepatic stellate cell (HSC) activation leading to high expression of multiple pro-angiogenic and mitogenic growth factors as well as excessive collagen deposition and scar formation (fibrosis) which culminate in the formation of abnormal cirrhotic liver nodules[73,74]. This microenvironment promotes genomic instability, deregulated growth and ultimately HCC development[73].

HCC patients with underlying liver disease (cirrhosis) have a poorer prognosis and generally more treatment-refractory disease than non-cirrhotic patients[75]. Sorafenib treatment of HCCs grown in cirrhotic mouse livers has been reported to reduce liver-associated fibrosis, but increase tumour-associated fibrosis and desmoplasia, which was found to be mediated by hypoxia-induced SDF-1α/CXCR4 pathway[76]. SDF-1α may also stimulate myeloid cell infiltration into the tumour and confer therapy resistance and immunosuppression[68]. Activated HSCs form fibrous septa and capsules and localize around vessels within HCCs[77] which may form physical barriers that reduce tumour cell invasion, drug penetration and immune cell infiltration[78]. Conceivably, fibrosis may restrict the ability of tumour cells to co-opt the vasculature.

Since human tumour xenografts necessitate an immunocompromised host, the effects of the immune system (particularly T and B cells for SCID mice) on tumour biology and therapeutic outcome cannot be evaluated. Angiogenesis and immunosuppression are two tightly linked processes in cancer. Independent of angiogenesis, VEGF stimulates the recruitment of myeloid-derived suppressor cells and regulatory T-cells, and inhibits dendritic cell maturation[79]. In contrast to other anti-angiogenic agents, sorafenib may further promote an immunosuppressive microenvironment through various mechanisms[68,79,80]. Taken together, lack of an intact host immune system or liver fibrosis/cirrhosis may

result in an exaggeration of the anti-tumour activity of sorafenib in mice. Other interactions between the immune system and the identified resistance mechanisms are unclear.

Based on these criticisms, it can be argued that the mechanisms of sorafenib resistance implicated in my studies may not be clinically applicable. This is a valid point particularly regarding tumour-dependent CYP3A4-mediated sorafenib metabolism, as such a phenomenon could be cell-line dependent. However the clinical relevance of many of my other findings are already established, namely: 1) declining sorafenib levels was a finding initially observed in patients[45,46,81,82]; 2) vessel co-option has been reported in several human tumour specimens including HCC[83,84]; 3) increased infiltrative growth during anti-angiogenic treatment has been observed in human glioblastoma[85]; and 4) reversible or unstable forms of drug resistance has been described in human cancers[86].

## FUTURE DIRECTIONS

One next step in this research program would be to study another animal model of HCC for evidence of co-option. For this, testing sorafenib in immunocompetent animal models of HCC would be advisable particularly since TKIs including sorafenib can act on the immune system to increase immunotolerance[80]. Various models exist, including genetically engineered animal models, as well as hepatotoxin-induced HCC models, which have the further advantage of modeling liver damage prior to HCC development[87]. Syngeneic orthotopic mouse models of HCC were recently reported, which involves orthotopic implantation of murine HCC cells or Cre-recombinase inducible Stk4(-/-)Stk3(F/-) mice on a background of carbon-tetrachloride-induced liver fibrosis[88]. Such models would be important to validate resistance mechanisms and test novel therapies in HCC.

The main critical path forward involves determining whether vessel co-option is a mechanism of sorafenib resistance, especially acquired resistance, in HCC patients, and whether the same mechanisms apply to other cancers beyond the liver and liver metastases. Clinical studies should be undertaken that analyze the early and late effects of anti-angiogenic therapy on tumour perfusion (ie. in responding patients and at disease progression) to test for a lack of rebound angiogenesis during progression/resistance. In this regard so few studies have been undertaken in anti-VEGF treated patients, let alone in HCC. While it is very difficult to obtain biopsy specimens from advanced HCC patients, the only (or at least best) way to definitely determine whether a switch to co-option has occurred is by evaluating histological specimens taken before and during the course of therapy. Other cancers where biopsies are easier to obtain may be more suitable for this type of research.

A final important research direction, as mentioned above, is the need to find molecular markers of co-opted vessels in the tumour, and from such studies, develop new therapies that can target these vessels. As mentioned above, this could include combination with other therapeutic modalities such as chemotherapy, immunotherapy and novel targeted agents.

## CONCLUSIONS

Over three decades since Judah Folkman put forward the seemingly straight-forward theory that tumours depend on angiogenesis for growth and progression[89], anti-angiogenic therapies have become highly integrated into standard-of-care treatments for many malignancies. However the true extent of dependency of tumours on angiogenesis is being questioned based on the modest effects these agents have on PFS or OS. The nature of the drug resistance phenotype when the genetically stable tumour

endothelium is targeted is also highly debated, with the appropriate next-line therapeutic options equally unclear.

My work builds on that of others which are beginning to address these questions. A poor response to standard treatment with an anti-angiogenic agent may not indicate resistance to therapy, but rather that the optimal dose has not been attained. The finding that many tumour types, including small, early-stage lesions, may not require induction of the angiogenic switch but survive by co-opting the host tissue vasculature[83] could help explain the poor response of certain tumours to angiogenesis inhibitors and the lack of efficacy of such agents in early stage adjuvant treatment settings[90]. Though vessel co-option has not historically been recognized in HCC, it may be an important factor in the natural course of this disease[84] and have heightened importance during angiogenic inhibition. Finally, not only is anti-angiogenic therapy resistance not like conventional drug resistance, but many other cancer therapies share reversible, unstable resistance properties with anti-angiogenic agents. These properties include activity with extended and repeated use in settings of disease progression where further treatment might typically be considered futile.

In conclusion, we have observed that reversible sorafenib resistance is mediated by at least two resistance mechanisms – a pharmacokinetic mechanism involving a decline in drug exposure levels over time and a switch to vessel co-option of host liver vessels. These results have important implications on how anti-angiogenic agents can be used more effectively – through either continuing angiogenesis inhibition beyond progression, dose individualizing therapy, or combination with new therapies that prevent or target vessel co-option in addition to angiogenesis. Furthermore, reversible resistance may be a small part of a larger and perhaps under-appreciated concept in oncology that offers more therapeutic options when treatments begin to fail.

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