Effect of Src Kinase Inhibition on Metastasis and Tumor Angiogenesis in Human Pancreatic Cancer

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Chapter 1

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Chapter 2

ABSTRACT

Tumor angiogenesis is a process that requires migration, proliferation, and differentiation of endothelial cells. We hypothesized that decrease in pancreatic tumor growth due to inhibition of src activity is associated with the inability of src kinase to trigger a network of such signaling processes, which finally leads to endothelial cell death and dormancy of angiogenesis.

The therapeutic efficacy of Src kinase inhibitor AZM475271 was tested in nude mice orthotopically xenografted with L3.6pl pancreatic carcinoma cells. No liver metastases and peritoneal carcinosis were detected and a significant effect on the average pancreatic tumor burden was observed following treatment with AZM475271, which in turn correlated with a decrease in cell proliferation and an increase in apoptotic endothelial cells. AZM475271 was shown to significantly inhibit migration of human umbilical vein endothelial cells in an *in vitro* Boyden Chamber cell migration assay. In a rat aortic ring assay we could demonstrate as well inhibition of endothelial cell migration and sprouting following therapy with Src kinase inhibitor at similar doses. Furthermore, we could show reduced proliferation of HUVECs determined with the TACS MTT Cell Viability Assay Kit. The blockade of Src kinase significantly reduced the level of VEGF in L3.6pl medium, the effect which was found also in the cell culture supernate from HUVECs. Inhibition of Src kinase by AZM475271 also showed prevention of survival signalling from VEGF and EGF receptors. Treatment with AZM475271 resulted in VEGF – dependent inhibition of tyrosine phosphorylation of FAK. HUVECs were also examined using propidium iodide staining for cell cycle analysis by FACS. Inhibition of src kinase promoted HUVEC apoptosis in a dose-dependent manner.

Taken together, our results suggest that the Src kinase inhibitor AZM475271, in addition to its effects on tumor cells, suppresses tumor growth and metastasis *in vitro* and *in vivo* potentially also by anti-angiogenic mechanisms.

Chapter 3

INTRODUCTION

3.1 Pancreatic cancer

3.1.1 Epidemiology and clinical characteristics

Pancreatic cancer is one of the most fatal malignancies in humans and continues to be a major medical challenge in the western world. The incidence rate of about 10 per 100,000 equals the mortality rate, underscoring the devastating nature of this disease (Van Cutsem et al., 2004). The five-year survival rate for pancreatic cancer is very low, less than 3% (Jemal et al., 2002), the median overall survival is 3-6 months with a 12-month survival rate of 10% (Bramhall et al., 1995). The very poor prognosis is due to late symptoms and the inability to detect this disease at early stages. Even with current diagnostic modalities, the inaccessible location of the pancreas often makes the diagnosis of pancreatic cancer a real challenge. Pancreatic cancer spreads early and most of the patients show liver or lymph node metastases at the time of diagnosis. More than 80% of patients have disease-related symptoms, such as jaundice, pain, asthenia, weight loss, and poor performance status (Cascinu et al., 1999). Even though painless jaundice occurs in about 50% of patients with respectable lesions of the pancreatic head, in general early symptoms are non-specific, and jaundice may also occur as a late symptom of large tumors of the body of the gland. Pain is the most frequent symptom and is present in 80% of patients with locally advanced tumors and metastatic disease (Van Cutsem et al., 2004). Nearly 90% of pancreatic neoplasms are adenocarcinomas, arising from the exocrine ductal system. They are most often multicentric and 75% are located in the head of the pancreas. The etiology of pancreatic cancer is not well understood but risk factors such as smoking, chronic pancreatitis and positive family history are implicated (Ahlgren, 1996; Hruban et al., 1998; Li et al., 2004). To date, surgical resection is the only potentially curative treatment, but the majority of patients are not surgical candidates due to advanced disease or significant co-morbidity (Brand 2001). For over 80% of pancreatic carcinoma patients, palliative treatment protocols represent the only reasonable therapeutic option (ASCO, 2006 Gastrointestinal Cancers Symposium).

This information indicates that current interventions to prevent, diagnose, and cure the disease are far from satisfactory. Only the understanding of the molecular mechanisms of pancreatic carcinogenesis will provide novel clues for preventing, detecting and ultimately curing patients with this life-threatening disease.

3.1.2 Standard chemotherapy of pancreatic cancer

Gemcitabine (2',2'-difluoro-2'-deoxycytidine) represents the current standard drug for cytotoxic therapy of advanced pancreatic cancer. Gemcitabine is a pyrimidine analog with a wide spectrum of antitumor activity (Abbruzzese, 1996). It is metabolized intracellulary by nucleoside kinases to the active species gemcitabine-diphosphate (dFdCDP) and gemcitabine-triphosphate (dFdCTP). Incorporation of dFdCTP into DNA is responsible for the cytotoxic effects of gemcitabine, *via* inhibition of DNA synthesis, DNA repair and ultimately *via* induction of apoptosis. Moreover, gemcitabine is a radio-sensitizing agent which acts specifically in the S and G1/S phase of the cell cycle.

5-Fluorouracil (5-FU), a fluoropyrimidine analogue, is also one of the most commonly used anticancer drugs for the treatment of pancreatic cancer. Some studies reported that the cytotoxicity of fluoropyrimidines is mediated, in large part, by inhibition of the thymidylate synthase (Carreras et al; 1995). Thymidylate synthase (TS) is the critical target for fluoropyrimidine cytotoxic drugs. It provides the sole *de novo* source of thymidylate for DNA synthesis. TS catalyzes the methylation of dUMP (deoxyuridine-5'-monophosphate) to dTMP (deoxythymidine-5'-monophosphate), which forms a tight-binding covalent complex with TS (Danenberg, 1977).

Based on the results from different randomized trials, first-line and second-line gemcitabine treatment in patients with advanced pancreatic cancer reveal significant advantages compared to 5-FU treatment regarding disease stabilization, overall survival and clinical benefit response measured as a decrease in pain, functional impairment and weight loss (Burris et al., 1997; Carmichael et al., 1996; Casper et al., 1994; Rothenberg et al., 1996). Several studies have focused on gemcitabine plus cisplatin (Philip, 2002; Lund et al., 1996). However, a very recent meta-analysis of larger Phase II and Phase III trials did not show a significant advantage for gemcitabine-cisplatin combinations over gemcitabine monotherapy, regarding 6-month survival rate, clinical benefit and toxicity (Xie et al., 2006). Similary, combination of gemcitabine and irinotecan resulted in superior response rates but not in improvement of progression-free or overall survival (Rocha Lima et al., 2002, 2004; Stathopoulos et al., 2003). Other combinations with gemcitabine, such as the addition of docetaxel, pemetrexed and exatecan mesylate, have proved disappointing in larger trials (Jacobs, 2002; Kindler 2002; O'Reilly et al., 2004; Richards et al., 2004; Stathopoulos et al., 2001).

Taken together, gemcitabine represents the current standard drug for therapy of advanced pancreatic cancer, and surgical patients might benefit from adjuvant gemcitabine therapy. New

conventional cytotoxic agents and other gemcitabine combinations might improve survival, but the improvement is likely to be small. Therefore, there is an urgent need for a better understanding of the mechanisms that contribute to pancreatic cancer growth and metastasis and for the design of more effective therapies for it.

3.1.3 Molecular mechanisms of pancreatic carcinogenesis

Over the past few years, our knowledge of the pathogenesis of pancreatic cancer has advanced significantly because of a rapid increase in our understanding of the molecular biology of it. Like many other malignant diseases, pancreatic cancer results from the accumulation of inherent and acquired genetic and epigenetic alterations. The multigenic nature of most pancreatic cancers is reflected by abnormalities of three broad classifications of genes: oncogenes, tumor suppressor genes and genomic maintenance genes (Sohn et al., 2000; Sakorafas et al., 2001). Accumulated alterations of such genes are believed to occur over a predictable time course. Based on the understanding of the histological and molecular genetic profiles of pancreatic cancer, investigators have developed a progression model that describes pancreatic ductal carcinogenesis: the pancreatic ductal epithelium progresses from normal epithelium to increasing grades of pancreatic intraepithelial neoplasia to invasive cancer (Hruban et al., 2000).

The majority of pancreatic cancers occur sporadically and have been fairly well characterized at the genetic level. Pancreatic cancer pathogenesis is apparently involved in the activation of several oncogenes and/or inactivation of various tumor suppressor genes (Sohn et al., 2000; Kern, 2000). Since the identification of the first notable genetic alteration of the K-*ras* oncogene, there has been an explosion in our understanding of pancreatic cancer genetics (Sohn et al., 2000; Kern, 2000). For examples, more than 85% of pancreatic cancers have an activating point mutation in the K-*ras* gene at a very early stage of development (Almoguera et al., 1988). Also, the tumor suppressor gene *p16* is inactivated in about 95% of pancreatic cancers, and inactivation typically occurs late in pancreatic carcinogenesis. *TP53*, a well-characterized tumor suppressor gene located on chromosome 17p, is the second most frequently inactivated gene. Furthermore, *DPC4* or *SMAD4* is inactivated in 55% of pancreatic adenocarcinomas. Both *TP53* and *DPC4* inactivation are late events in pancreatic tumorigenesis. Other less common genetic alterations continue to be described in pancreatic cancer. In a comprehensive mutational analysis of 42 pancreatic cancers, Rozenblum et al. (1997) found that all of the tumors harboured mutations of the K-*ras* oncogene. The individual mutational frequency of the tumor suppressor genes *p16*, *TP53*, *MADH4* and *BRCA2* was 82, 76, 53 and 10%, respectively. Presumably, these

alterations promote cellular proliferation, suppress apoptotic pathways, and facilitate tumor angiogenesis, invasion and metastasis.

However, the molecular mechanisms that link these genetic changes with the aggressive nature of pancreatic cancer remain poorly understood. These genetic alterations are generally perceived to eventually lead to various abnormalities in the expression and functions of a variety of growth factors and their receptors and to affect their downstream signal transduction pathways involved in the control of cell proliferation and differentiation (Li et al., 2004; Sakorafas et al., 2001; Kern, 2000; Korc, 2003). For example, pancreatic cancer cells overexpress many families of growth factors and their receptors, including epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and its receptor and plateled-derived growth factor (PDGF), as well as many cytokines, such as transforming growth factor (TGF)-β, tumor necrosis factor-α, interleukin (IL)-1, IL-6 and IL-8, which enhances mitogenesis (Korc, 2003; Xie et al., 2001, 2003 and 2004). Pancreatic cancer also exhibits loss of responsiveness to various growth-inhibitory signals, such as members of the TGF-β family (Korc, 2003). The disturbed production and function of growth-promoting and -inhibiting factors are believed to confer a tremendous survival and growth advantage to pancreatic cancer cells, as manifested by the development of angiogenic, invasive and metastatic phenotypes that are resistant to all conventional treatments. The clinical importance of the findings described above is underscored by numerous experimental and clinical observations (Korc, 2003). For example, the concomitant presence of EGF receptor (EGFR) and either EGF or TGF-β in the cancer cells is associated with disease progression and decreased survival. EGFR blockade with an anti-EGFR antibody attenuates pancreatic tumor growth, and inhibition of EGFR tyrosine kinase activity suppresses pancreatic angiogenesis (Bruns et al., 2000). These findings are among the many that support the hypothesis that dysregulated production and function of growth factors has an important role in pancreatic cancer.

3.2 The role of angiogenesis in local and systemic tumor growth

3.2.1 Tumor angiogenesis

Tumor angiogenesis is the formation of new blood vessels from existing vessels and new circulating endothelial progenitor cells from bone marrow (Folkman, 1990). In 1971, Folkman proposed that tumor growth and metastasis are angiogenesis-dependent, and hence, blocking angiogenesis could be a strategy to arrest tumor growth (Folkman, 2000). This possibility stimulated an intensive search for pro- and anti-angiogenic molecules. In 1976, Gullino showed

that cells in pre-cancerous tissue acquire angiogenic capacity on their way to becoming cancerous (Gullino, 1978). He proposed that this concept be used to design strategies to prevent cancer (Gullino, 1978), a hypothesis later confirmed by genetic approaches (Hanahan et al., 2000).

3.2.2 The angiogenic switch

It is now widely accepted that the "angiogenic switch" is "off" when the effect of pro-angiogenic molecules is balanced by that of anti-angiogenic molecules, and is "on" when the net balance is tipped in favour of angiogenesis (Hanahan et al., 2000; Bouck et al., 1996). Various signals that trigger this switch have been discovered (Table 3.1). Pro- and anti-angiogenic molecules can emanate from cancer cells, endothelial cells, stromal cells, blood and the extracellular matrix (Fukumura et al., 1998). Their relative contribution is likely to change with tumor type and tumor size, tumor growth, regression and relapse.

3.2.3 Formation of tumor vessels

Tumor vessels develop by sprouting or intussusception from pre-existing vessels. Circulating endothelial precursors, shed from the vessel wall or mobilized from the bone marrow, can also contribute to tumor angiogenesis (Asahara et al., 2000; Rafii, 2000). In addition, tumor vessels lack protective mechanisms that normal vessels acquire during growth. For example, they may lack functional perivascular cells, which are needed to protect vessels against changes in oxygen or hormonal balance, provide them necessary vasoactive control to accommodate metabolic needs, and induce vascular quiescence (Benjamin et al., 1999). Finally, the vessel wall is not always formed by a homogenous layer of endothelial cells (Jain, 1988). Instead, it may be lined with only cancer cells or a mosaic of cancer and endothelial cells.

Activators	Function
VEGF family members	Stimulate angio/vasculogenesis and permeability
VEGFR, NRP-1	Integrate angiogenic and survival signals
Ang1 and Tie2	Stabilize vessels, inhibit permeability
PDGF-BB and receptors	Recruit smooth muscle cells
TGF-β and receptors	Stimulate extracellular matrix production
FGF, HGF	Stimulate angiogenesis
Integrins	Receptors for matrix macromolecules and proteinases
Plasminogen activators, MMPs	Remodel matrix, release and activate growth factors

Inhibitors	Function
VEGFR-1, soluble VEGFR-1	Sink for VEGF, VEGF-B
Ang2	Antagonist of Ang1
TSP-1,-2	Inhibit endothelial migration, growth, adhesion and survival
Angiostatin	Suppresses tumor angiogenesis
Endostatin	Inhibits endothelial survival and migration
Platelet factor-4	Inhibits binding of bFGF and VEGF
Prolactin	Inhibits bFGF/VEGF
Interferons and interleukins	Inhibit endothelial migration; downregulate bFGF

Table 3.1 Angiogenesis activators and inhibitors

3.2.4 Structure and function of tumor vessels

Tumor vessels are structurally and functionally abnormal: their walls have numerous “openings” and a discontinuous or absent basement membrane. In addition, the endothelial cells are abnormal in shape, growing on top of each other and projecting into the lumen. These defects make tumor vessels leaky (Hobbs et al., 1998; Hashizume et al., 2000; Dvorak et al., 1999).

3.3 Angiogenic phenotype of pancreatic cancer

Although pancreatic cancer is not a grossly vascular tumor, it often exhibits enhanced foci of endothelial cell proliferation. Moreover, several (Korc, 2003; Seo et al., 2000; Linder et al., 2001; Fujioka et al., 2001; Mirecka et al., 2001; Stipa et al., 2002; Niedergethmann et al., 2002; Khan et al., 2002; Kuwahara et al., 2003; Shibaji et al., 2003; Karademir et al., 2000; Ikeda et al., 1999; Ellis et al., 1998) but not all (Fujimoto et al., 1998) studies have reported a positive correlation between blood vessel density and disease progression in cases of pancreatic cancer, supporting the important role of angiogenesis in this disease.

At the molecular level, numerous factors have been shown to be involved in pancreatic cancer angiogenesis. Among this growing list of growth factors, VEGF is believed to be critical for pancreatic cancer angiogenesis (Korc, 2003; Xie et al., 2004). Several studies have shown that VEGF expression correlates with microvessel density and disease progression (Korc, 2003; Seo et al., 2000; Niedergethmann et al., 2002, 2000; et al., 1999; Knoll et al., 2001). Additionally, VEGF-c is overexpressed in pancreatic cancer and correlated with enhanced lymph node metastasis (Tang et al., 2001). Moreover, pancreatic cancers overexpress several other mitogenic growth factors that are also angiogenic, such as EGF, TGF-α, HGF, FGFs and PDGF-β (Korc, 2003; Balaz et al., 2001). Together, these factors may produce mitogenic activity in an autocrine and paracrine fashion, promoting pancreatic tumor cell growth and angiogenesis and eventually enhancing pancreatic tumor invasion and metastasis.

3.4 Therapeutic angiogenesis

Inhibition of neo-angiogenesis is a new and attractive target for tumor therapy, since it theoretically offers the hope of long-term control of tumor progression. Antiangiogenic therapy offers a number of potential benefits including lack of resistance to some agents, synergistic interaction to other modalities, lack of significant toxicity compared with conventional agents, and a potent antitumor effect. Administration of angiogenesis inhibitors might keep the tumor and its metastases dormant (rather than killing it), and co-administration of cytotoxic drugs might kill it (Teicher et al., 1995; Satoh et al., 1998).

Consistent with the roles of the factors described above, anti-angiogenic therapies have been demonstrated to suppress tumor growth in animal models of pancreatic cancer (Bruns et al., 2000-2004).

Based on successful preclinical data, several anti-angiogenic agents alone or in combination with conventional therapies are now in clinical trials (Table 3.2; www.cancertrials.nci.nih.gov). Numerous studies have been conducted to investigate the effects of different receptor tyrosine kinase inhibitors (Table 3.2).

Drug	Mechanism
Phase I	
PTK787/ZK2284	Blocks VEGF-receptor signaling
Matuzumab	EGFR1 monoclonal antibody
Erlotinib	EGFR kinase inhibitor
Vatalanib	VEGFR kinase inhibitor
SU6668	Blocks VEGF-, FGF- and PDGF-receptor signaling
Phase II	
Imatinib	PDGFR kinase inhibitor
Cetuximab	EGFR1 monoclonal antibody
Erlotinib	EGFR kinase inhibitor
Phase III	
SU5416	Blocks VEGF-receptor signaling
Thalidomide	Unknown
AG3340	Synthetic MMP inhibitor
Interferon-α	Inhibition of bFGF and VEGF production
IM862	Unknown mechanism
Marimastat	Synthetic MMP inhibitor

Table 3.2 Angiogenesis inhibitors in clinical trials for cancer (including pancreatic cancer)

However, as tumors grow, they begin to produce a wider array of angiogenic molecules. Therefore, if only one molecule (for example, VEGF) is blocked, tumors may switch to another molecule (for example, bFGF or IL-8). Thus we may require a cocktail of antibodies/inhibitors.

Several oncogenes and their intracellular protein products such as v-*src* and v-*ras* induce the up-regulation of angiogenic factors such as VEGF, EGF, bFGF and PDGF and increase the production of cytokines and proteolytic enzymes (Mukhadopathyay et al., 1995; Jiang et al., 1997; Kerbel et al., 1998; Okada et al., 1998; Kypta et al., 1990; Arbiser et al., 1997). In this respect, targeting intracellular proteins might be a promising approach in angiogenesis therapy of pancreatic cancer.

3.5 Kinases of the Src family nonreceptor protein tyrosine kinases

One of the potential intracellular therapeutic targets receiving considerable recent attention is activation of c-Src, a nonreceptor protein tyrosine kinase. In pancreatic adenocarcinomas, src is activated in more than 70% of primary tumors (Coppola, 2000).

3.5.1 The discovery of c-Src proto-oncogene

In 1911, Peyton Rous first described a viral agent capable of inducing tumors in chickens providing evidence for the transmissible nature of cancer (Rous, 1911). This seminal work went largely unappreciated and uncorroborated until the 1950s, when tumor cells were shown to arise from infection with the Rous sarcoma virus (Rubin, 1955). This finding was confirmed when temperature sensitive activated v-Src mutants failed to transform cells at non-permissive temperatures, demonstrating a requirement for the active virus in cellular transformation (Martin, 1970). In the 1970s, Brugge and colleagues isolated and identified v-Src as the transforming protein of the oncogenic Rous sarcoma virus utilizing tumor bearing rabbit serum (Brugge et al., 1977). Additional experiments demonstrated that the viral Src gene (v-Src) has a highly conserved and ubiquitously expressed cellular homologue, c-Src, which is present in normal cells (Stehelin et al., 1976). Src was not only the first proto-oncogene identified, it was also the first demonstrated to possess intrinsic protein kinase activity (Collett et al., 1978; Levinson et al., 1978) spawning both a search for similar protein tyrosine kinases and investigations into the role of Src in regulating cellular behavior. The last two decades have witnessed an explosion in Src research, including the recent development of selective small molecule inhibitors that target Src.

3.5.2 Src family members

The non-receptor protein tyrosine kinase Src is the prototypical member of a kinase family that includes Yes, Fyn, Lyn, Lck, Hck, Fgr, Yrk, Frk and Blk. This group is collectively known as the Src family kinases (SFKs), and, in contrast to receptor protein tyrosine kinases (VEGFR, EGFR, PDGFR), is not comprised of transmembrane proteins (Neet et al., 1996).

Tyrosine kinases catalyze the transfer of phosphate from ATP to a tyrosine residue of specific cell protein targets.

3.5.3 Structural organization of Src proteins

As a result of mutational studies and structural modeling based on crystallography data, the structure of Src has been well characterized.

In vertebrates the proteins of the Src family have similar structure (Brown et al., 1996). The proteins of this group, ranging in molecular mass from 52 to 62 kD, comprise six distinct functional domains (Fig. 3.1 and 3.2): Src homology domain 4 (SH4), a unique domain, SH3 domain, SH2 domain, a catalytic domain (SH1), and a C-terminal regulatory region.

The SH4 domain is a region containing from 15 to 17 amino acid residues which comprises signals for modification with fatty acids (Rech, 1993). The glycine at position 2 is myristylated, thus binding protein tyrosine kinase (PTK) to the cell membrane. Nonmyristylated Src molecules do not bind to membranes. On the other hand, some Src molecules carrying this modification can be found unlinked in the cytosol. Myristylation probably does not guarantee association of the protein with the membrane. In addition to myristylation signals, the Src SH4 domain contains basic amino acid residues which are substrates for post-translational palmitylation. Only myristylated molecules are palmitylated and consequently this process probably occurs on the membrane. Palmitylation is a reversible process. Regulated depalmitylation and repalmitylation

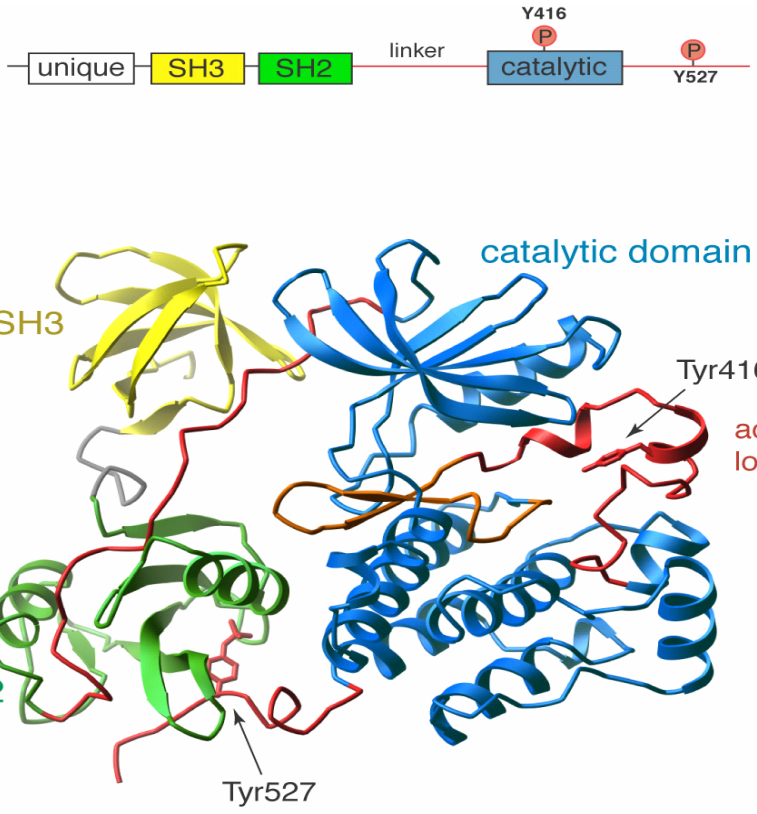


Figure 3.1 Ribbon diagram illustrating the structure of human Src. Key phosphorylation sites are included.

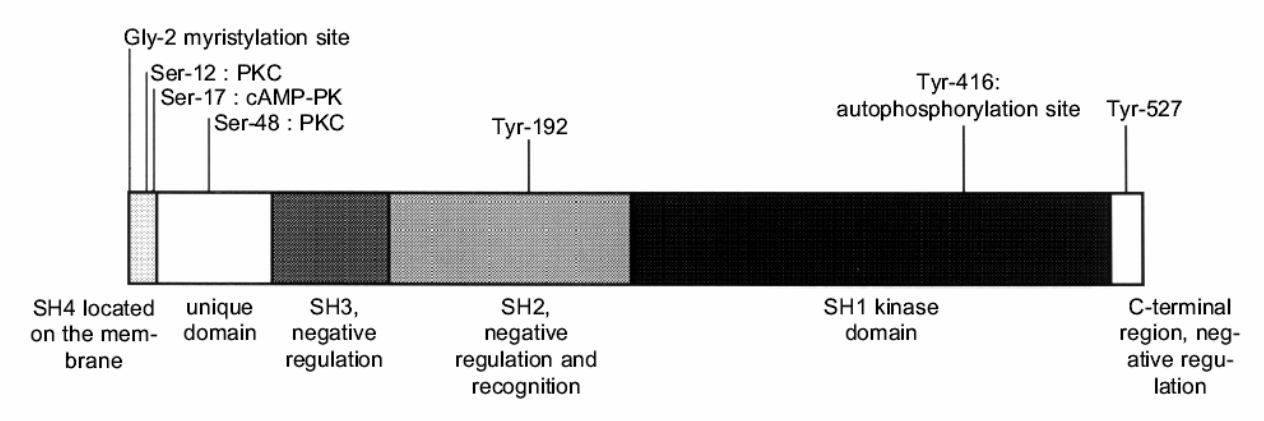


Figure 3.2 Domain structure of Src protein. Key phosphorylation sites are included.

may be a mechanism for the changing of the Src family kinases localization in response to corresponding stimulation (Kaplan et al., 1988; Pellman et al., 1985).

The unique domain (amino acid residues 18-84) is specific for each Src family protein. This region is suggested to be responsible for specific interaction of the PTK with particular receptors and protein targets (Thomas et al., 1997). At the same time, Src is a single member of the Src kinase group which is phosphorylated at Thr-34, Thr-46, and Ser-72 by a cyclin-dependent kinase (Cdc2) and cyclin B complex in M phase (Superti-Furga et al., 1995). The effect of this phosphorylation is unclear. Moreover, the unique domain contains the protein kinase C (PKC) binding sites – Ser-12 and Ser-48 – which are phosphorylated in the cells during PKC activation.

The SH3 domain (amino acid residues 85-140) is necessary for interactions with protein substrates and it also ensures the intramolecular bindings controlling catalytic activity, protein localization in the cell, and association with protein targets (Pawson, 1995). The SH3 domain of Src has a globular structure, one side of which is slightly hydrophobic and contains a cluster of acidic residues. This domain binds to the proline-rich regions of PTK substrates. All known SH3 ligands carry a consensus sequence PXXP. Amino acid residues adjacent to pro-line determine specificity of SH3 domains (Riskles et al., 1995). The SH3 ligands may bind to this domain both in NH_2–COOH and COOH–NH_2 orientation (Yu et al., 1994).

The SH3 sequences as well as the SH2 and the catalytic domains shown below, have been found in cellular proteins of different classes.

The SH2 domain is a second modulating region (amino acid residues 141-260), which controls the range of proteins interacting with the Src family kinases. The SH2 domains of different PTK recognize a short amino acid sequence carrying phosphotyrosine. From three to five amino acid

residues following tyrosine determine the specificity of individual SH2 domains (Songyang et al., 1993). In kinases of the Src family this region is more conserved than the SH3 domain and can be tightly bound with specific proteins phosphorylated by tyrosine. The SH2 domains of Src and Lck kinases carry deep hydrophobic pockets for interactions of amino acid Ile at position pY + 3. Some proteins interacting with Src contain an optimum binding structure, pYEEI. However, not all proteins binding to the SH2 domain of Src possess such phosphorylated sequence.

A mutation in the SH2 domain in the region of amino acid residues from 142 to 169 leads to cell transformation (Raymond et al., 1987). This mutation probably affects Src binding to substrates, cytoskeletal proteins in particular (see below).

The kinase domain (amino acid residues 265-516) is found in all proteins of the Src family as well as in other PTK. It is responsible for tyrosine kinase activity and plays a crucial role in substrate specificity (Hesketh, 1995). Certain amino acid residues within this domain are identical in all kinases and involved in ATP binding and the phosphotransferase reaction (Hughes, 1996).

The kinases of the Src family may bind some substrates after phosphorylation, thus promoting phosphorylation of other sequences of one or several neighboring substrate molecules (Mayer et al., 1995). A strict specificity towards Tyr but not towards Ser or Thr is due to the close proximity of a conserved loop present in all tyrosine kinases (FP^{425}IKWTA in Src) to the main chain of the substrate. Proline facilitates binding to the phenylalanine ring of tyrosine, but is ineffective in binding substrates carrying serine or threonine.

Phosphorylation of Tyr-416 stimulates complete activation of Src and provides a binding site for SH2 domains of other cellular proteins. The elimination of Leu-516, highly conserved in all protein tyrosine kinases and located in the catalytic domain, interferes with the transforming activity of p60 v-Src (Yaciuk et al., 1986).

The C-terminal region (amino acid residues from 517 to ~536) plays a significant role in regulation of Src kinase activity. All kinases of the Src family have a C-terminal region of 15-19 amino acid residues with tyrosine at the constant position surrounded by conserved amino acids (Tyr-527 in Src). It has been shown that elimination of phosphotyrosine from the normal Src increases its kinase activity (Schwartzberg, 1998). Phosphorylation of the C-terminal Tyr inhibits kinase activity by more than 98% and suppresses all stimulating effects caused by phosphorylation of Tyr-416 in the catalytic domain (Van Hoek et al., 1997).

3.5.4 Structural differences between v-Src and c-Src proteins

The main difference between v-Src and c-Src is found in the structure of their C-terminal regions (Fig. 3.3). The last "tail" 19 amino acids of c-Src contain Tyr-527, which plays a regulatory role controlling kinase activity (Yaciuk et al., 1986; Dorai et al., 1991). In v-Src these 19 amino acids are replaced by 12 amino acids present in all known RSV strains. The only exception is v-SrcLM whose atypical structure is associated with a decreased metastatic potential of transformed cells (Tatosyan et al., 1996). It has been recently demonstrated that in metastases of human colon cancer, the c-Src protein has mutations just in this region. It should be mentioned that now this is the most reliable example of the role of mutations in the src gene in human carcinogenesis (Irby et al., 1999).

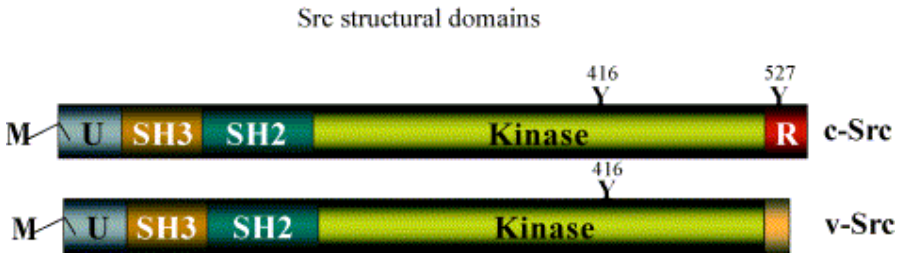


Figure 3.3 Domain structure of cellular (c-) and viral (v-) Src

3.5.5 Regulation of Src kinases

The SH2 and SH3 domains play a key role in regulation of catalytic activity of the Src family kinases. X-Ray analysis has demonstrated how intramolecular interactions between SH2 and SH3 domains stabilize inactive conformational structure of Src kinases. Both domains are adjacent to the kinase domain from the side opposite to the catalytic cleft. The SH3 domain interacts with the catalytic domain and linker sequences located between SH2 and catalytic domains (Fig. 4) (Xu et al., 1999). The SH2 domain interacts with phosphotyrosine at position 527 localized in the C-terminal region of the protein. Tyr-527 in c-Src, as well as the corresponding tyrosine residues in other PTK are the primary phosphorylation sites *in vivo*. This base is phosphorylated by the cytoplasmic kinase Csk (Nada et al., 1991). The loss of Tyr-527 or its dephosphorylation leads to stimulation of Src catalytic activity. This conclusion is based on a number of experimental data: the substitution of Tyr-527 by another amino acid residue constitutively activates c-Src (Kmiecik et al., 1987); this region is absent from the v-Src protein (Reynolds et al., 1987); the inhibition of the *csk* gene activity stimulates activity of PTK of the

Src family (Imamoto et al., 1993). Therefore, it has been suggested that phosphorylation of the C-terminal tyrosine by Csk kinase provides intramolecular interaction of this region with the SH2 domain thus preserves the Src protein in a closed inactive form.

Mutations in the SH3 domain also lead to activation of Src kinases, although the role of the SH3 domain in inhibition of protein enzymatic activity is obscure. The kinase domain apparently remains accessible even in "closed" conformation, namely, when the SH2 domain is associated with phosphotyrosine at position 527 (Fig. 3.4). The SH3 domain forms an independent intramolecular contact with the N-terminal fragment of the kinase domain. Accordingly, kinase inactivation may result from the formation of a rigid structure stabilized by double bonds between the SH2 and SH3 domains and the catalytic region of the protein. Such structure prevents any movement inside the kinase domain. Mutations in any of the interacting regions of the Src protein disrupt the rigid structure of the molecule; this, in turn, destabilizes other intramolecular interactions. Consequently, both the SH2 and SH3 domains regulate kinase activity by intramolecular contacts. Abnormal interactions may be the main activation

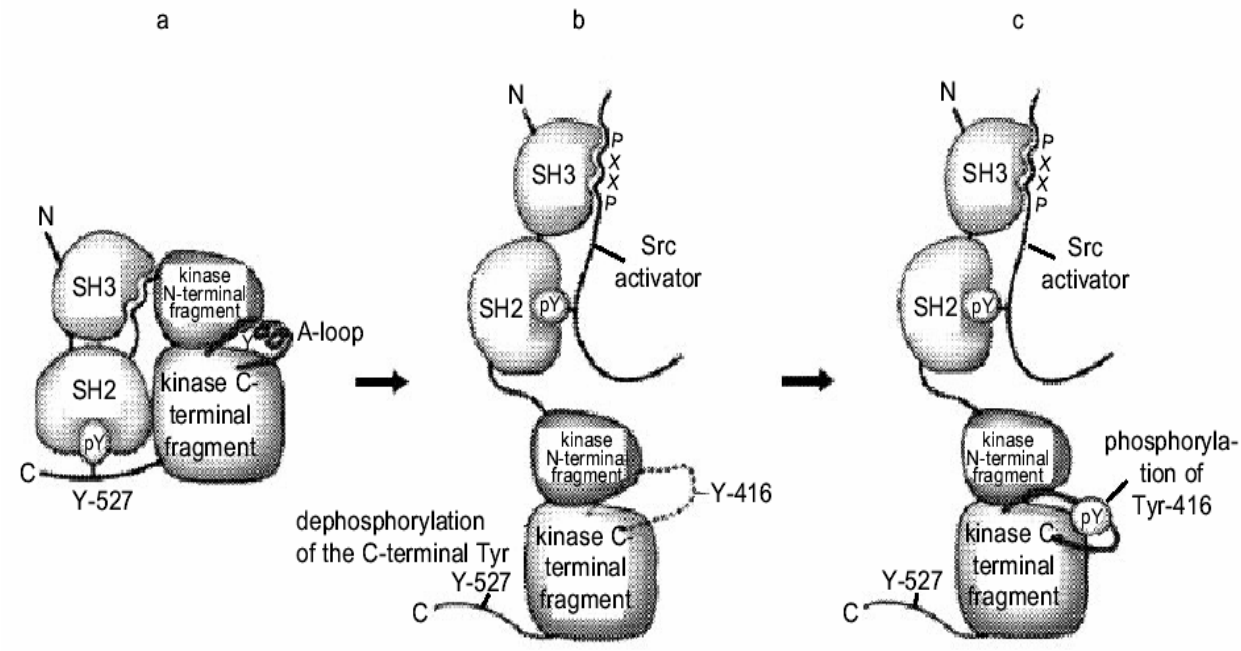


Figure 3.4 Regulation of Src activity:
a) Closed autoinhibited state;
b) Open intermediate state induced either by interactions of the SH3 and SH2 domains with the Src protein partners or by dephosphorylation of the C-terminal Tyr-527. This makes Tyr-416 accessible for phosphorylation;
c) Open activated form with phosphorylated Tyr-416

mechanism of the Src proteins. It cannot be excluded that these domains play a similar role in regulation of kinase activity in proteins that lack phosphotyrosine in the C-terminal region (Fps, Abl) (Pawson et al., 1992). It should be noted that the above-mentioned mechanism not only precisely regulates kinase activity of the Src proteins, but controls the interactions of SH2 and SH3 domains with other molecules, thus providing different levels of Src regulation.

As shown above, the regulation of Src activity occurs at two sites, the modification of each of them leads to opposite results. The phosphorylation of Tyr-416 in the activating loop of the kinase domain activates the enzyme, while the phosphorylation of the C-terminal Tyr-527 causes its inactivation. Different regulatory elements controlling Src activity affect only particular regions in the kinase domain. These effectors contain amino acid residues involved in catalysis or substrate binding (Sichei et al., 1997). They may be the activating loop (amino acid residues 404-432), the catalytic loop (the region around the amino acid at position 382), and the C-helix (the region around the amino acid at position 310). The modulation of their position and conformation by phosphorylation and interaction with regulatory subunits may control catalytic activity.

In vivo the Src kinase can be phosphorylated only at one of two tyrosine residues. The model for Src tyrosine kinase activation includes three subsequent stages (Fig. 3.4).

The activating loop plays a central role in regulating kinase activity. Its phosphorylation at Tyr-416 in Src (or homologous amino acid residues in other tyrosine kinases) is necessary for complete activation of most kinases studied so far. In the absence of phosphorylation, the activating loop acquires different conformations, which often inhibit protein–protein interactions. A nonphosphorylated activating loop can inhibit kinase activity either directly, disturbing the region involved in activation, or indirectly, conferring a specific conformation, which prevents substrate binding. Conversely, in the phosphorylated state, the conformation of the activating loop is similar in all known kinases. In this active conformation the loop forms a part of the site recognized by the substrates (Xu et al., 1997).

3.5.6 Src substrates

The proteins tyrosine phosphorylated as a result of *src* gene function are briefly summarized in Table 3.3.

3.5.7 Role of Src in regulation of cellular processes

c-Src is a multifunctional protein involved in the regulation of a variety of normal processes, including proliferation, differentiation, survival, motility, angiogenesis, and functions of fully

differentiated cells (Thomas and Brugge, 1997). To carry out these activities, c-Src interacts with numerous cellular factors, including cell surface receptors (EGF family, CSF-1, PDGF, and FGF receptors, as well as integrins, cell-cell adhesion molecules, etc. [Biscardi et al., 2000; Irby and Yeatman, 2000; Owens et al., 2000; Moro et al., 2002]), steroid hormone receptors (Migliaccio et al., 1996, 2000; Boonyaratanakornkit et al., 2001), components of pathways regulated by heterotrimeric G proteins (Luttrell et al., 1999; Ma et al., 2000), STATs (Silva et al., 2003), focal adhesion kinase (FAK) (Kaplan et al., 1994), the adaptor proteins p130Cas (Burnham et al., 2000) and Shc (Sato et al., 2002), and many others. Each of these partners represents a different class of proteins and functions in unique signaling pathways for

Protein target	Function	Src domain responsible for binding
p85 subunit of PI-3 kinase	noncatalytic PI-3 kinase subunit	SH3, SH2
RasGAP	GTPase activator Ras	
SHPTP2/Syp	phosphotyrosine phosphatase	
Shc	binds Grb2, an adaptor protein participating in translocation of the factors involved in nucleotide metabolism (GEF) to the membrane where Ras is located, thus activating a signal pathway	
PLCγ	PI-specific phospholipase C	
p62	RasGAP-associated protein	
p190	GTPase activator Rho	
Tensin	binding to actin, integrin-dependent signal transduction mechanism	
Vinculin	binding to actin, integrin-dependent signal transduction mechanism	
Cortactin	binding to cortical actin	SH2
Talin	binding to actin and integrin, integrin-dependent signal transduction mechanism	
Paxilline	integrin-dependent signal transduction mechanism	SH3
AFAP110	associated with actin filaments	SH3, SH2
FAK	tyrosine kinase, binding to integrin and integrin-dependent signal transduction mechanism	SH2
β1-Integrin	formation of adhesive complexes and signal transduction mechanism	
$p130^{CAS}$	integrin-dependent signal transduction mechanism	SH2
$p120^{CAS}$	catenin, cell adhesion	
β-Catenin	binding to integrin, intercellular adhesion	
γ-Catenin	cell adhesion	
Sam68	RNA binding	SH2, SH3
Calpactin I – annexin II	Ca^{2+}-phospholipid binding	

Table 3.3 Target proteins for Src kinases

which the molecular nature and biological consequences of the interactions with c-Src have been investigated. The interactions between c-Src and other molecules can be found in Figure 3.5. Src is also implicated in invasion and angiogenesis, the latter property of which will be extensively discussed below (3.7).

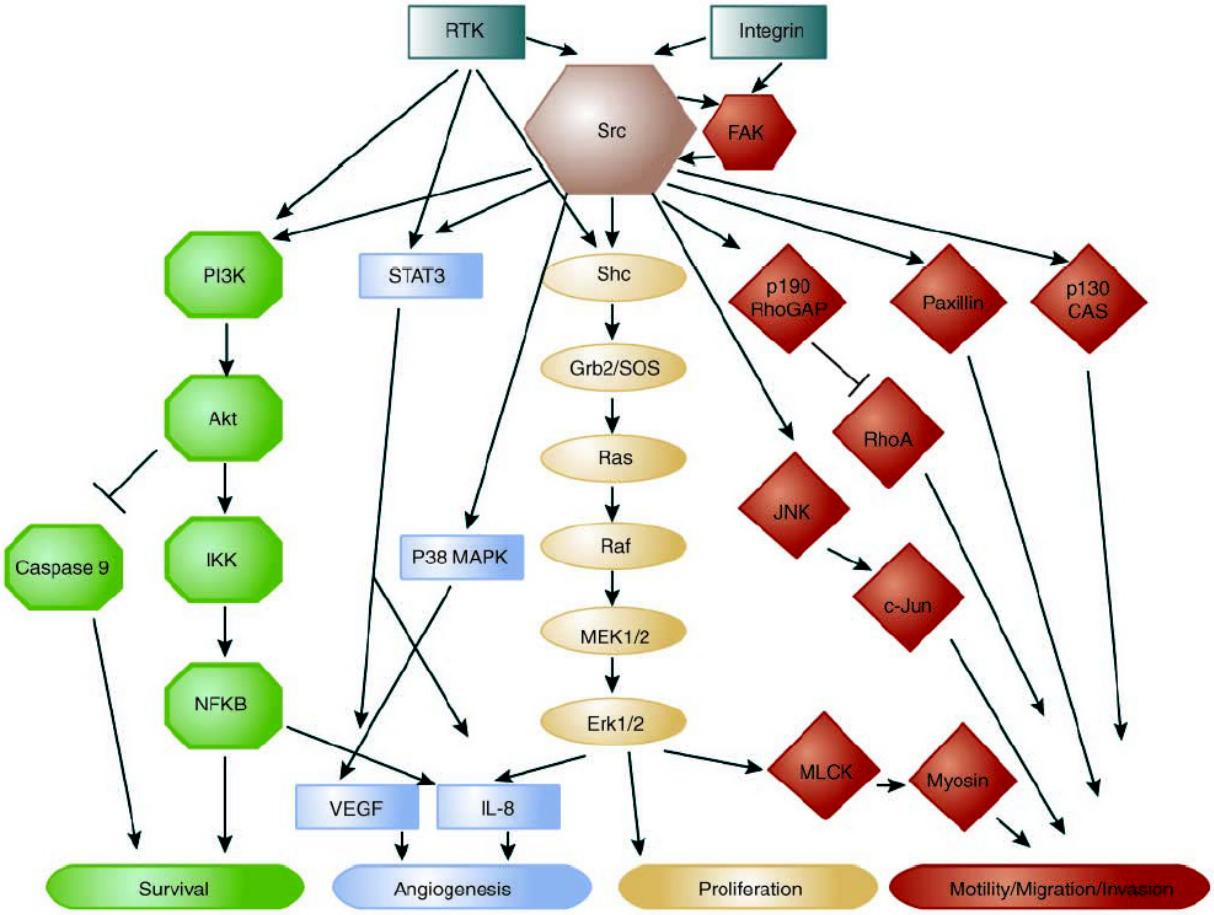


Figure 3.5 c-Src is a protein involved in the regulation of a variety of biological processes, including proliferation, differentiation, survival, motility, angiogenesis, and functions of fully differentiated cells.

RTK, receptor tyrosine kinase; PI3K, phosphatidylinositol 3-kinase; STAT3, signal transducers and activators of transcription 3; IKK, InB kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinase; NF-κB, nuclear factor-κB; FAK, focal adhesion kinase.

3.6 Src activation and tumor progression

The likely regulators, or types of regulation, which can lead to Src's conformational "opening" and activation that might occur in cancer cells are shown in Figure 3.6.

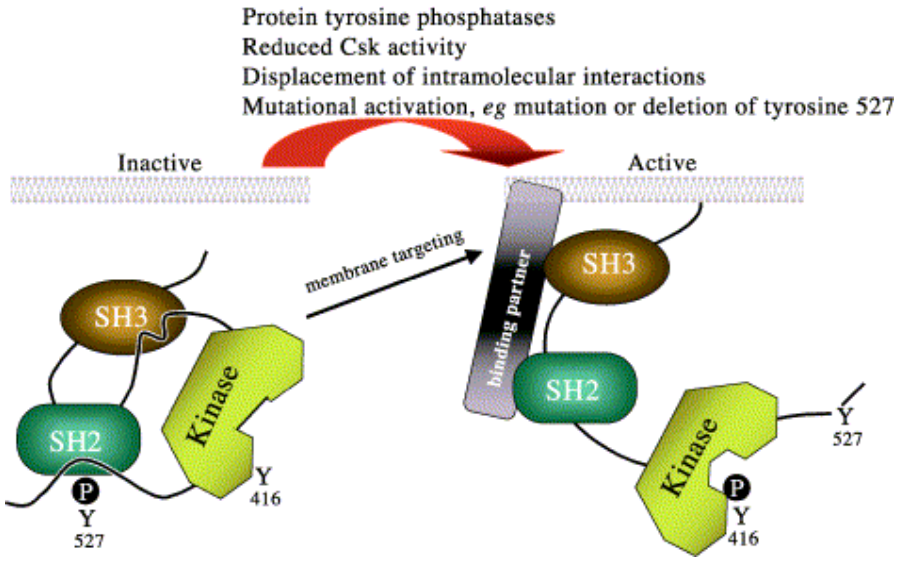


Figure 3.6 Regulation of Src activity in tumor cells

The relationship between Src activation and cancer progression appears to be significant. The evidence underlying this hypothesis is largely based on the observation that both Src protein levels and, to a greater degree, Src protein kinase activity, are frequently elevated in human neoplastic tissues when compared to adjacent normal tissues. Moreover, these levels appear to increase with the stage of disease. Similarly, increased Src protein kinase activity has been observed in numerous human cancer cell lines derived from these tumors.

Src kinase activity, from 4 – 20-fold higher than normal tissues, has been found in human mammary carcinomas (Egan et al., 1999; Jacobs and Rubsamen, 1983; Muthuswamy et al., 1994; Verbeek et al., 1996). Similarly, cell lines derived from these tumors display up to a 30-fold elevation in Src activity.

The c-Src proto-oncogene has frequently been implicated in the initiation and progression of human colon cancer (Bolen et al., 1987a; Cartwright et al., 1989, 1990, 1994; Weber et al., 1992). Src activity is increased 5 – 8-fold in the majority of colon tumors.

Elevated Src protein levels and/or kinase activity has been reported in lung (50 – 80%) (Mazurenko et al., 1992), neural (23/27 neuroblastomas, 3/3 retinoblastomas) (Bjelfman et al.,

1990; Bolen et al., 1985), ovarian (Budde et al., 1994; Wiener et al., 1999), esophageal (3 – 4-fold increases in activity in Barrett's esophagus and 6-fold elevations in adenocarcinomas) (Kumble et al., 1997) and gastric cancers (Takeshima et al., 1991), as well as melanoma (Bjorge et al., 1996) and Kaposi's sarcoma (Munshi et al., 2000).

Src activity has recently been studied in pancreatic cancer. Lutz et al. (1998) examined pancreatic ductal carcimonas as well as pancreatic cell lines for elevated Src protein levels and kinase activity. Src protein levels were elevated in 13/13 pancreatic cancers and in 14/17 pancreatic cell lines. Kinase activity was only detectable in cancer cells and this activity did not correlate with either the c-Src or Csk protein levels.

3.7 Src and tumor angiogenesis

There is a significant amount of data supporting the influence of Src on tumor angiogenesis and metastasis.

Src activation is required for hypoxia-mediated expression of VEGF (Mukhopadhyay et al., 1995). More recently, Trevino et al. (2005) have shown that Src regulates both constitutive and growth factor-induced VEGF and interleukin-8 expression and that Src activation up-regulates VEGF mRNA transcription by activation of signal transducers and activators of transcription 3, which forms a complex with hypoxia-inducible factor-1 and other factors on the VEGF promoter (Gray et al., 2005). The experiments by Ellis et al. (1998) demonstrated that antisense Src strategy was effective in reducing both constitutive and hypoxic-induced VEGF production in a human colon carcinoma cell line. In addition, how Src links between VEGF stimulation and the control of cell survival remains to be determined. It has been shown that receptor tyrosine kinases activate Src by autophosphorylation of tyrosine residues that function as docking sites for the SH2 domain of Src kinases on the receptor itself or by phosphorylation of docking proteins. Binding of the SH2 domain of Src to tyrosine phosphorylation site on VEGFR or on a docking protein releases Src from the autoinhibited state and enables the catalytic activity to be stimulated by autophosphorylation of a key tyrosine residue in the activation loop of the catalytic core. Once activated, Src could link VEGF-stimulation with the PDK/PKB signaling cassette leading to stimulation of endothelial cell survival and angiogenesis.

There is also evidence that Src cooperates with the EGFR in growth signaling (Roche et al., 1995; Wilson et al., 1989). Fibroblasts that overexpress Src display an enhanced mitogenic response to EGF, an effect that is independent on Src's myristylation and catalytic activity (Luttrell et al., 1988). However, the molecular understanding of synergy between Src and the

EGFR in mitogenic signaling is much less advanced than for PDGFR (Mori et al., 1993; Broome et al., 1999; Stover et al., 1996), although it seems likely that Src and activated EGFR form a complex and that EGF induces Src activation (Biscardi et al., 1999).

Further, Karni et al. have demonstrated the ability of constitutively active Src to induce HIF-1alpha under normoxic conditions (Karni et al., 2002) suggesting that Src activation, regardless of mechanism, can augment VEGF production and angiogenesis. Ongoing work from this laboratory has demonstrated a role for Src in regulating VEGF expression in other tumor types including pancreatic cancer, where Src activation of the PI3kinase/Akt signaling pathway is required (Summy et al., 2006). As Src is a regulator of multiple signal transduction pathways, it should not be surprising that Src is important in regulating other pro-angiogenic factors such as bFGF, and IL-8.

In addition to regulating VEGF and bFGF expression, Src regulates responses to these factors in both tumor and endothelial cells. Elicieri and co-workers have shown both bFGF and VEGF are able to induce Src activation in avian endothelial cells (Elicieri et al., 1999). Additionally, overexpression of the dominant negative Src induces apoptotic cell death in the VEGF-treated endothelial cells suggesting a survival function for Src during VEGF-induced angiogenesis. Similar results were obtained when utilizing a virus that encodes Csk, a protein tyrosine kinase that inhibits Src activity by phosphorylating Tyr-530 in the c-terminal tail.

Overexpression of a kinase-inactive Src also resulted in reduced phosphorylation of paxillin and cortactin, suggesting a role for Src in actin cytoskeletal rearrangement and migration (Kilarski et al., 2003).

As in tumor cells, activated Src promotes a mesenchymal-like phenotype in endothelial cells, increasing migratory potential of these cells as well (Potter et al., 2005).

There is convincing evidence that the linked activities of Src and focal adhesion kinase (FAK) control cell migration. In support of a role for Src, cells expressing kinase-defective v-Src have enlarged focal adhesions and their migration is suppressed (Fincham et al., 1998). Src and FAK co-localize at integrin adhesion sites and cooperate with growth factor receptors, such as EGF and PDGF to induce signaling pathways that control diverse aspects of cell behavior, including growth, survival and migration (Figure 3.7).

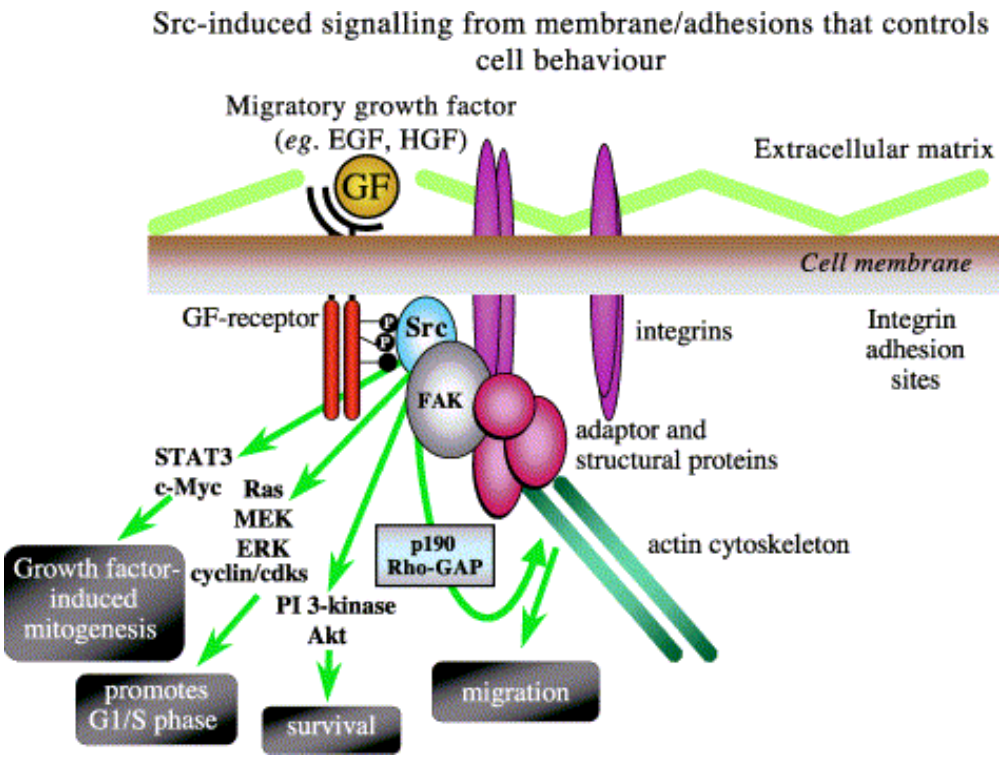


Figure 3.7 Src, FAK and adhesion turnover during fibroblast migration (Frame, 2002)

Current results suggest Src activation in tumor cells indirectly regulates Src activity in endothelial cells. Increased Src activity in the tumor cells increases VEGF expression, resulting in increased binding to VEGF receptors on endothelial cells. This process then leads to association of Src with these receptors, increasing Src activity in endothelial cells as well. This continuing cycle promotes Src-mediated increases in migratory potential and permeability of endothelial cells and facilitates tumor cell extravasation (Figure 3.8). The importance of targeting tumor-associated endothelial cells for therapeutic efficacy has been highlighted from recent work on inhibitors of receptor tyrosine kinases in clinical trial (Table 3.2) (Yokoi et al., 2005; Thaker et al., 2005; Yazici et al., 2005; Lev et al., 2005, Yigitbasi et al., 2004). Inhibitors of Src thus have the potential to interfere with this cycle by affecting biological functions in both tumor and tumor-associated endothelial cells that contribute to metastasis.

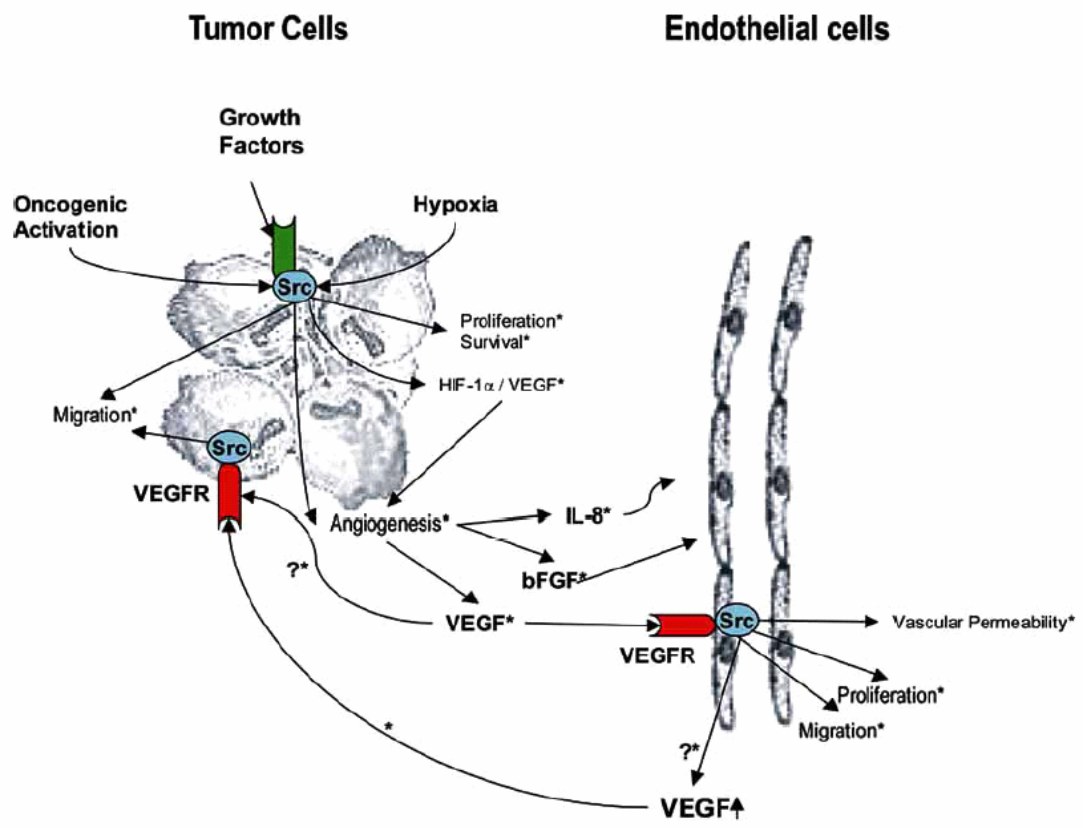


Figure 3.8 Model by which Src activity mediates biologic functioning of tumor cells and endothelial cells, and promotes intercellular signaling to promote tumor progression and metastasis. * Indicates the signaling molecules and/or cellular functions potentially affected by Src kinase inhibition.

Chapter 4

THESIS PROPOSAL

The prevalence of activated Src in cancer indicates that this protein plays a significant role in the progression of many cancers, but since all of them (and specially pancreatic cancer) are angiogenesis dependent, the main goal of the work described in this thesis was to demonstrate the role of Src family protein kinases in pancreatic tumor progression with special emphasis on angiogenic regulation, and how Src inhibitors may affect this process (Figure 4).

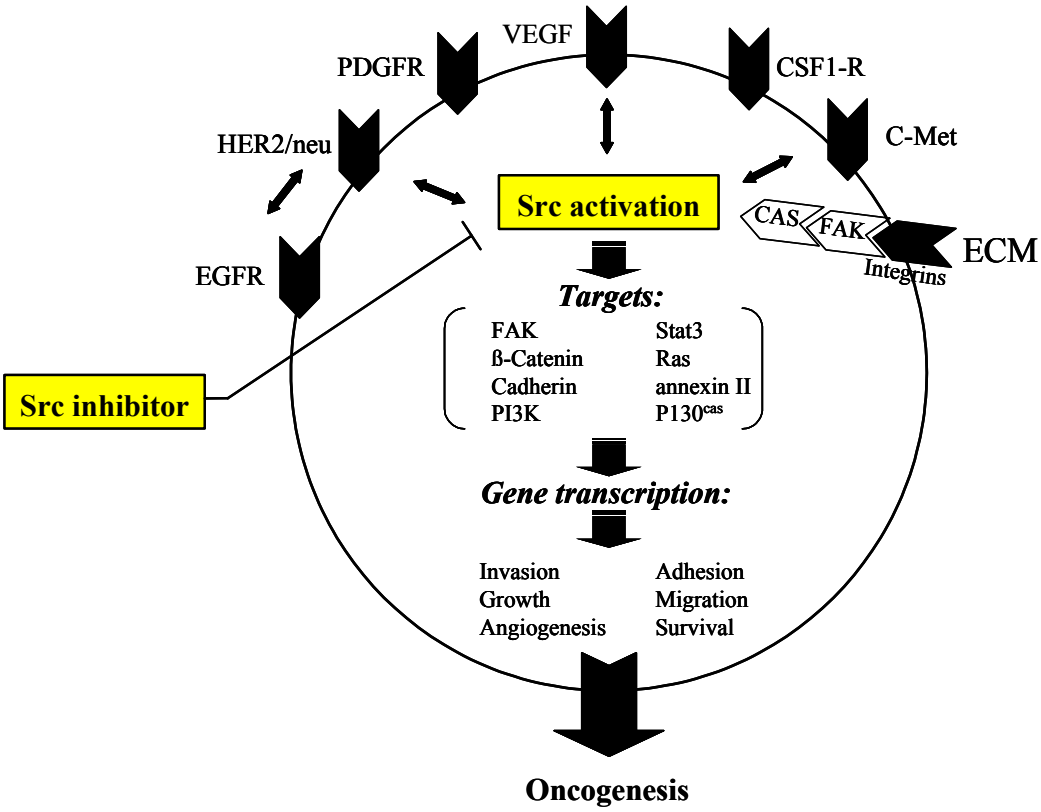


Figure 4 Thesis proposal: inhibition of Src tyrosine kinase as an anti-angiogenic therapy of human pancreatic cancer

Chapter 5

MATERIALS AND METHODS

5.1 Materials

5.1.1 Laboratory equipment

0.45 µm sterile filters	BD Falcon, France
–20ºC Freezer	Siemens AG, Germany
4ºC Fridge	Siemens AG, Germany
5 ml FACS tubes	BD Biosciences, Belgium
–80ºC Freezer	Heraeus, Hanau, Germany
Automatic pipettes	Gilson, Middleton, WI, USA
AxioCam MRc5 camera	Carl Zeiss GmbH, Germany
Blotting chamber	Bio-rad, Munich, Germany
BD Biocoat™ Matrigel invasion chamber	BD Biosciences, Belgium
Cell counting chambers	Bürker-Türk, Germany
Centrifuges	Eppendorf, Germany
CO_2 Incubators	Heraeus, Rodenbach, Germany
Digital Precision Scale	KERN & Sohn GmbH, Germany
Embedding cassettes	Leica Microsystems GmbH, Germany
Fluorescence-activated cell sorter (FACS)	Becton Dickinson, USA
Gel electrophoresis systems	Bio-rad, Munich, Germany
Heating block	Biometra, Germany
Hybond-P membrane	Amersham Biosciences, Germany
Hyperfilm	Amersham Biosciences, Germany
Kinetic microplate reader	υ max, USA
Laminar flow hoods	Heraeus, Hanau, Germany
Liquid nitrogen tank	MVE, New Prague, MN, USA
Microscopes	Carl Zeiss GmbH, Germany
pH-meter	WTW, Weilheim, Germany
Positively-charged superfrost slides	Menzel-Glaeser, Germany
Rocking Platforms	Biometra GmbH, Göttingen, Germany
Rotary microtome	Leica Microsystems GmbH, Germany
Semi-Dry Electrophoretic Transfer Cell	Bio-rad, Munich, Germany

Sterile cell scrapers	TPP, Switzerland
Sterile cryotube vials	TPP, Switzerland
Sterile tissue culture plastic flasks and plates	NUNC, Denmark
Sterile tissue culture plastic tubes, dishes, pipets	TPP, Switzerland
Tissue embedding console system	Sakura, Torrance, CA, USA
Vortex	IKA Works, Wilmington, NC, USA
Water bath	GFL, Burgwedel, Germany
X-ray film developing machine	AGFA, Germany

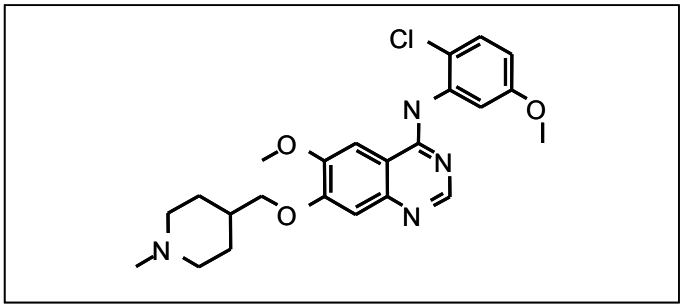
5.1.2 Chemical reagents and other research solutions

2-Mercaptoethanol	Merck, Darmstadt, Germany
3,3′,5,5′-Tetramethylbenzidine tablets (TMB)	Sigma-Aldrich GmbH, Germany
30% Acrylamide/Bis Solution	Roth, Karlsruhe, Germany
4',6-diamino-2-phenylindole (DAPI)	Molecular Probes, USA
D,1-Dithiothreitol (DTT)	Sigma-Aldrich GmbH, Germany
Acetic acid 96%	Sigma-Aldrich GmbH, Germany
Ammonium Persulfate	Sigma-Aldrich GmbH, Germany
Aprotinin	Roche Diagnostics GmbH, Germany
ATP	Sigma-Aldrich GmbH, Germany
Bovine serumalbumin (BSA)	Sigma-Aldrich GmbH, Germany
Dimethylsulfoxide (DMSO)	Sigma-Aldrich GmbH, Germany
EDTA	Sigma-Aldrich GmbH, Germany
Protein G Sepharose 4 fast flow	Amersham Biosciences, Germany
Ethanol	Merck, Darmstadt, Germany
Glycerol	Merck, Darmstadt, Germany
Glycine for electrophoresis	Merck, Darmstadt, Germany
HEPES	NeoLab GmbH, Germany
Isopropanol	Merck, Darmstadt, Germany
Leupeptin A	Roche Diagnostics GmbH, Germany
Magnesium Chloride	Merck, Darmstadt, Germany
Methanol	Merck, Darmstadt, Germany
Nonidet P-40	Roche Diagnostics GmbH, Germany
Phenylmethylsulfonyl fluoride (PMSF)	Sigma-Aldrich GmbH, Germany
Phosphate citrate buffer with Sodium Perborate	Sigma-Aldrich GmbH, Germany

Poly (Glu, Tyr) sodium salt	Sigma-Aldrich GmbH, Germany
Ponceau S	Sigma-Aldrich GmbH, Germany
Pre-stained protein ladder	Invitrogen Corporation, Germany
Protease inhibitor cocktail tablets	Roche Diagnostics GmbH, Germany
Skim milk powder (blotting grade)	Sigma-Aldrich GmbH, Germany
Sodium Chloride	Merck, Darmstadt, Germany
Sodium Citrate	Sigma-Aldrich GmbH, Germany
Sodium Hydroxide	Sigma-Aldrich GmbH, Germany
Sodium Vanadate	Sigma-Aldrich GmbH, Germany
Sodiumdodecylsulfat (SDS)	Biorad, Munich, Germany
Sulphuric Acid	Merck, Darmstadt, Germany
Tetramethylethylendiamine (TEMED)	ICN Biomedicals Inc, Aurora, USA
Tris base	Sigma-Aldrich GmbH, Germany
Triton-X100	Sigma-Aldrich GmbH, Germany
Propidium Iodide	Sigma-Aldrich GmbH, Germany
Human fibronectin	Sigma-Aldrich GmbH, Germany
Trypan blue	Serva, Heidelberg, Germany
Tween 20	Merck, Darmstadt, Germany
Water for molecular biology	Maxim Biotech GmbH, Germany
Xylene	Merck, Darmstadt, Germany

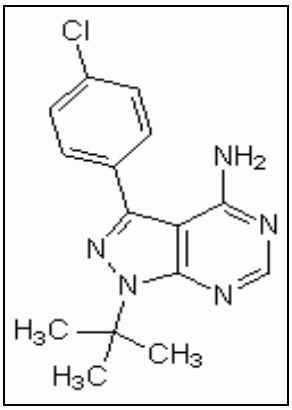
5.1.3 Drugs

AZM475271 (Scheme 5.1) is a novel anilinoquinazoline inhibitor of c-Src. AZM475271 was synthesized and kindly provided by AstraZeneca Pharmaceuticals (Macclesfield, United Kingdom). For *in vivo* administration, AZM475271 was dissolved in Tween 20 diluted 1:100 in NaCl, for *in vitro* applications the AZM475271 stock was prepared in DMSO and stored at + 4°C.



Scheme 5.1 Structure of the novel anilinoquinazoline inhibitor of c-Src AZM475271

4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2, Calbiochem, San Diego, CA, USA) is a potent and selective inhibitor of the Src family of tyrosine kinases that is similar to PP1. For *in vitro* studies PP2 (Scheme 5.2) was used as a comparable chemotherapeutic agent. PP2 was supplied as a 3.33 mmol/L stock in DMSO and stored at + 4°C.



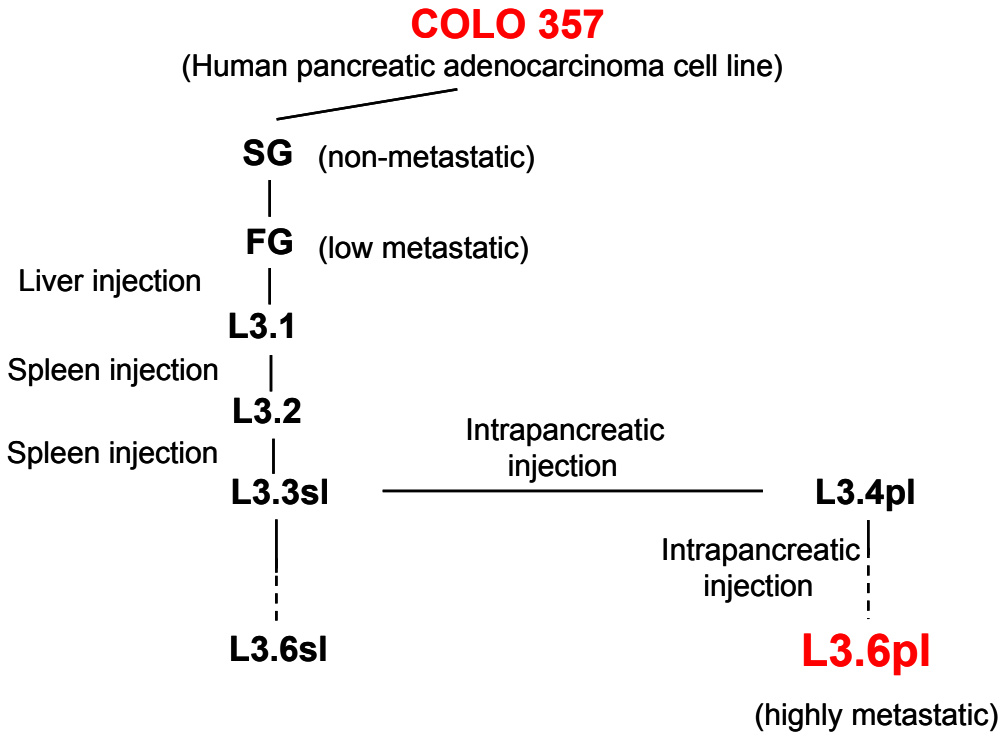
Scheme 5.2 Structure of the selective inhibitor of the Src family of protein tyrosine kinases PP2

5.1.4 Cell lines

Human highly metastatic pancreatic carcinoma cell line L3.6pl

For our *in vivo* and *in vitro* experiments we used variants of a human pancreatic adenocarcinoma cell line COLO 357. This cell line derived from a celiac axis lymph node that was partially replaced by neoplastic foci of well differentiated mucin-containing pancreatic ducts and it was originally isolated 1980 by a group from Colorado Morgan et al.

For *in vivo* selection of highly metastatic human pancreatic cancer cells, we injected cells from the original fast growing, low metastatic FG cell line derived from COLO 357 into the pancreas of nude mice. To produce liver lesions, human pancreatic cancer cells implanted into the pancreas must complete all the steps of the process. The lesions then are designated as spontaneous metastases. Spontaneous liver metastases were then harvested, established in tissue culture, and designated as L3.4pl. Cells harvested from these cultures were injected into the pancreas of another set of nude mice. Liver lesions were again isolated and cells were established in culture. After three such selection cycles, the cell line L3.6pl was established in culture (Fig. 5.1). Cytogenetic analysis confirmed the human origin of the cells.



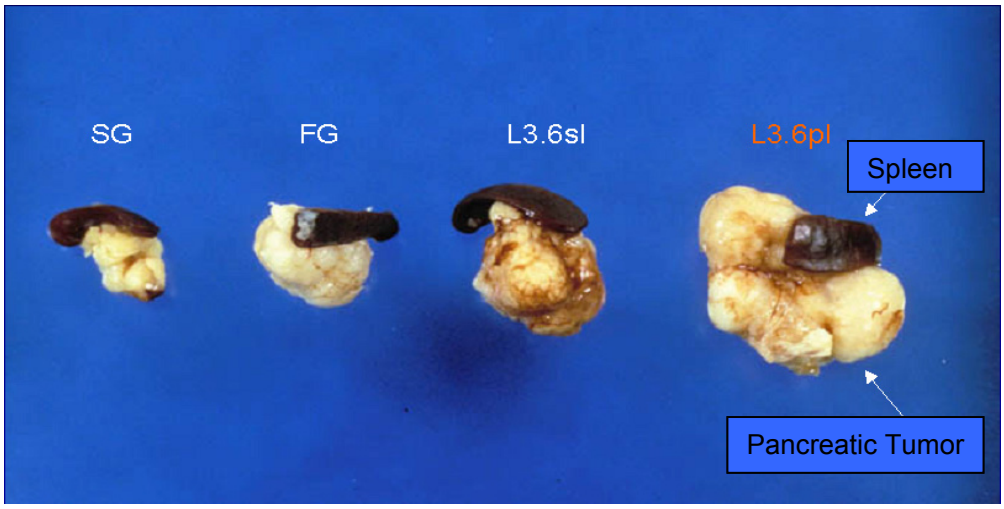


Figure 5.1 In vivo selection of metastatic human pancreatic cancer cells

The highly metastatic cell line L3.6pl showed an aggressive primary tumor growth in nude mice with spontaneous liver metastases (50% of animals) and spontaneous lymph nodes metastases (100% of animals), 50% of animals bearing L3.6pl cancer died after 36 days, whereas more then 60% of animals after the injection of parental FG cell line survived up to 100 days (Fig. 5.2). L3.6pl cells produced pro-angiogenetic factors such as VEGF, bFGF, as well as IL-8 and tumor samples presented with a high amount of microvascularisation. *In vitro* studies, IHC analyses and *in situ* hybridization of tumor samples showed a significantly low expression of E-cadherine and MMP-9/2 level in L3.6pl cells in contrast to the parental cell line (Bruns et al., 1999).

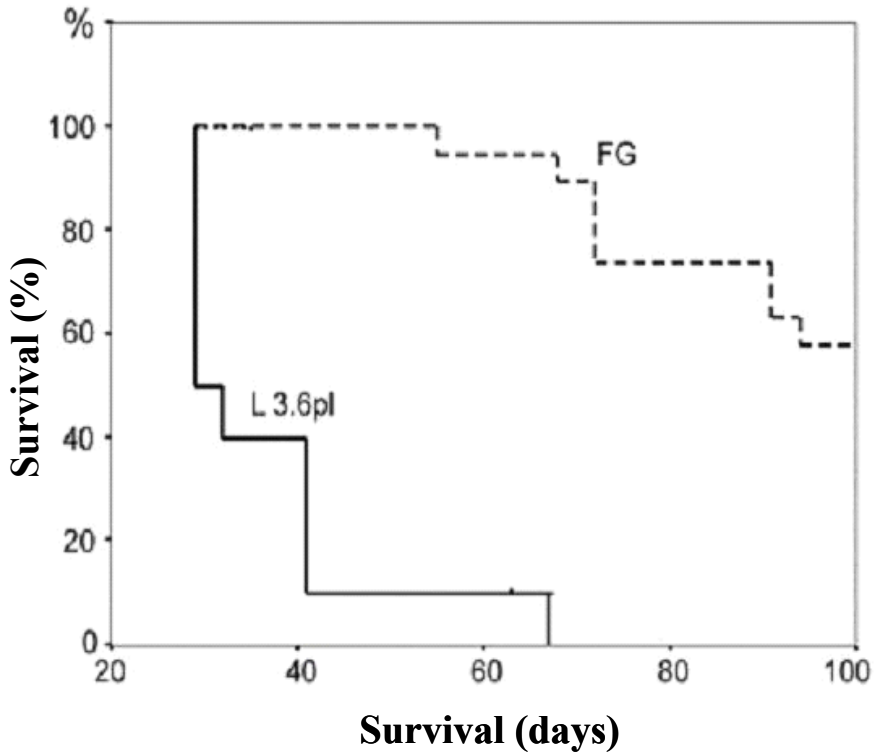


Figure 5.2 Kaplan-Meier survival test

Human umbilical vein endothelial cells (HUVECs)

HUVECs were purchased from PromoCell (PromoCell GmbH, Heidelberg, Germany).

5.1.5 Media and supplements

10-fold trypsin-EDTA	Biochrom AG, Berlin, Germany

10-fold vitamin solution	Biochrom AG, Berlin, Germany
Ampicillin	Gibco Invitrogen, Germany
Dulbecco´s Modified Eagle Medium (D-MEM)	Gibco Invitrogen, Germany
Endothelial Cell Growth Medium	PromoCell GmbH, Germany
Fetal Bovine Serum (FBS)	Biochrom AG, Berlin, Germany
Fetal Calf Serum	PromoCell GmbH, Germany
Human fibronectin	Sigma-Aldrich GmbH, Germany
Kanamycin	Gibco Invitrogen, Germany
L-Glutamin	Biochrom AG, Berlin, Germany
Nonessential amino acids (10-fold solution)	Biochrom AG, Berlin, Germany
Penicillin-streptomycin mixture	Biochrom AG, Berlin, Germany
Sodium Pyruvate	Gibco Invitrogen, Germany

5.1.5 Growth factors

bFGF	PromoCell GmbH, Germany
ECGS/H	PromoCell GmbH, Germany
Human recombinant EGF	R&D Systems, Wiesbaden, Germany
Human recombinant $VEGF_{165}$	R&D Systems, Wiesbaden, Germany
Hydrocortison	PromoCell GmbH, Germany
PDGF	PromoCell GmbH, Germany

5.1.6 Kits and other research products

Avidin-biotinylated Horseradish Peroxidase Complex (ABC Kit)	Vector Laboratories, CA, USA
BCA™ Protein Assay Reagent Kit	Pierce, Rockford, USA
DeadEnd™ Fluorometric TUNEL System	Promega, Madison, WS, USA
ECL® Western Blotting Detection System	Amersham Biosciences, Germany
Enhanced Chemiluminescence System	Amersham Biosciences, UK
Human VEGF Immunoassay Kit	R&D Systems, Minneapolis, MN, USA
Immunoprecipitation Kit	Sigma-Aldrich GmbH, Germany
Restore™ Western Blot Stripping Buffer	Pierce, Rockford, IL, USA
TACS MTT Cell Proliferation and	

Viability Assay Kit	R&D Systems, Minneapolis, MN, USA

5.1.8 Antibodies

Anti-FAK monoclonal	Cell Signaling Inc., Germany
Anti–phospho-tyrosine monoclonal, HRP	Santa Cruz Biotechnology, CA, USA
Anti-pTyr 576/577 FAK monoclonal	Cell Signaling Inc., Germany
Anti-ß-actin monoclonal	Sigma-Aldrich GmbH, Germany
Anti-v-src (Ab-1) monoclonal	Oncogene, San Diego, CA, USA
CD31/PECAM-1 rat anti-mouse monoclonal	Pharmingen, San Diego, CA, USA
Goat anti-rabbit polyclonal, biotinylated	Vector Laboratories, CA, USA
Goat anti–rabbit, HRP	Amersham, Freiburg, Germany
Ki-67-specific rabbit anti-human polyclonal	Zymed GmbH, Germany
Rabbit anti-rat polyclonal, HRP	DAKO, Germany

5.1.9 Animals for *in vivo* experiments

Male immunodeficient Balb/c nu/nu mice were purchased from Charles River Laboratories (Sulzfeld, Germany). The mice were housed and maintained in laminar flow cabinets under specific-pathogen-free conditions in facilities of Institute for Surgical Research (Munich, Germany). The mice were quarantined during the acclimatization period of at least a week. Food (Standard 1320 and 1430; Altromin, Lange, Germany) and acidified water (pH 2.5-3.0) were available *ad libitum*. Regular health checks were done. The mice were used in accordance with institutional guidelines when they were 8-12 week of age.

Male ACI rats were purchased from Harlan Winkelmann GmbH (Borchen, Germany). The rats were kept at two per cage under climatized conditions and were given standard food (Standard 1320 and 1430; Altromin, Lange, Germany) and water *ad libitum*. The rats were used in accordance with institutional guidelines when they were 6-8 weeks old.

All procedures were performed in accordance with current regulations and standards of the animal protect orders.

5.1.10 Materials used for *in vivo* studies

Atropine Sulfate	Braun AG, Germany
Cotton applicators	NOBA Verbandmittel GmbH, Germany
Cutasept® F	Bode Chemie, Hamburg, Germany
Disposable scalpels	Feather Safety Razor Co., Japan
Gauze swabs	NOBA Verbandmittel GmbH, Germany
Ketavet	Pfizer Pharmacia GmbH, Germany
Mouse gavage feeding needle	Kent Scientific, Torrington, USA
Pushbutton-controlled dispensing device	Hamilton Syringe Company, USA
Sodium Chloride Solution	Braun AG, Germany
Suture material	Braun AG, Germany
Syringes, needles	BD Biosciences, Spain
Xylazin (Rompun), 2%	Bayer HealthCare, Germany

5.1.11 Software

Adobe Acrobat 5.0	Adobe Systems Inc., USA
Adobe Acrobat Distiller 5.0	Adobe Systems Inc., USA
Adobe Photoshop 5.0	Adobe Systems Inc., USA
AxioVision 4.4	Carl Zeiss GmbH, Germany
SOFTmax 2.32	Molecular Devices Corp., USA
Cellquest Pro	Beckton Dickinson, USA
Image2PDF 1.4.5	Verypdf.com Inc., USA
ImageJ 1.33u	NIH
InStat 3.0	Graphpad Software, USA
Microsoft Office 2002	Microsoft Corporation, USA
Origin 6.0	Microcal Software Inc., USA
SPSS 8.0	SPSS STATISTICS Inc., USA
Windows XP Professional	Microsoft Corporation, USA
WinMDI 2.8	Joseph Trotter

5.1.12 Buffers

PBS Wash Buffer, 1X

140 mM *NaCl*

2.7 mM *KCl*

10 mM *Na_2HPO_4*

1.8 mM *KH_2PO_4*

High purity dH_2O, pH 7.4

PBS-T

1X PBS

0.1% *Tween-20*

Kinase Buffer, 1X

25 mM *Tris-HCl, pH 7.5*

5 mM *Beta-glycerophosphate*

2 mM *Dithiothreitol*

0.1 mM *Na_3VO_4*

10 mM *$MgCl_2$*

Src Kinase Dilution Buffer, 1X

100 mM *HEPES*

2 mM *Dithiothreitol*

0.2 mM *activated Na_3VO_4*

0.02% *BSA*

3 Unit/ml *Src Kinase*

RIPA Lysis Buffer, 1X

50 mM *Tris-HCl, pH 7.4*

150mM *NaCl*

1% *Nonidet P-40*

1 mM *activated Na_3VO_4*

1 mM *PMSF*

5 mM *EDTA, pH 8.0*

1 µg/ml *Aprotinin*

1 µg/ml *Leupeptin*

Filter sterilized

Protease and phosphatase inhibitors were added freshly before cell lysis.

Laemmli Buffer, 2X

0.5 M	*Tris-HCl, pH 6.8*
5%	*β-Mercaptoethanol*
0.1%	*Bromophenol Blue*
20%	*Glycerol*
4%	*SDS*

Towbin Transfer Buffer, 1X

25 mM	*Tris-HCl, pH 8.3*
192 mM	*Glycine*
20%	*Methanol*
High purity dH_2O	

Tris Buffered Saline (TBS) Buffer, 10X

1M	*Tris-HCl, pH 7.4*
1.5M	*NaCl*

TBS-T Buffer

1X TBS	
0.1%	*Tween-20*

SDS Electrophoresis Buffer, 10X

0.25 M	*Tris*
1.92 M	*Glycine*
1%	*SDS*

High purity dH_2O, pH 8.3 to 1000 ml

Solutions for Casting One 10% Separating and One Stacking Gel

	Separating gel, 10%	Stacking gel
H_2O	1.9 ml	1.4 ml
30% Acrylamide/Bis Solution	1.7 ml	0.33 ml
1.5M Tris-HCl (pH 8.8)	1.3 ml	-
1M Tris-HCl (pH 6.8)	-	0.25 ml
10% SDS	0.05 ml	0.02 ml
10% Ammonium Persulfate	0.05 ml	0.02 ml
TEMED	0.002 ml	0.002 ml

Milk Blocking Solution

5% w/v nonfat dry milk dissolved in TBS-T buffer

Ponceau S Staining Solution

0.5g of Ponceau S was dissolved in 1 ml of glacial acetic acid and the volume was adjusted to 100 ml with H_2O.

TMB Substrate Solution

One tablet of the HRP substrate TMB was dissolved in 100 µl of DMSO and added per 10 ml of phosphate citrate buffer with Sodium Perborate.

Nicoletti buffer (Propidium Iodide Staining)

0.1% *Sodium Citrate, pH 7.4*

0.1% *Triton X-100*

50 µg/ml *Propidium Iodide*

5.2 Methods

5.2.1 Cell biological methods

5.2.1.1 Cell culture techniques

The cells were cultivated under aseptic conditions in 75-cm^2 tissue culture flasks. All tissue culture flasks have caps with filters which allow gaseous exchange, allowing maintenance of correct pH (which is monitored by the colour of the phenol red present in the medium) and the right percentage of CO_2 (5%). The optimal atmosphere conditions are allowed by CO_2 incubator, which automatically control temperature and pCO_2; it operates with a try of water on the base in an attempt to maintain more than 98% relative humidity. Temperature of the incubator was set at 37°C and regularly controlled.

Human highly metastatic pancreatic carcinoma cells L3.6pl were maintained as monolayer cultures in Dulbecco´s Modified Eagle Medium (D-MEM) supplemented with 10% FBS, L-glutamine, sodium pyruvate, nonessential amino acids, vitamins, and penicillin-streptomycin mixture. The cultures were tested and found to be free of Mycoplasma and the following pathogenic murine viruses: reovirus type 3, pneumonia virus, K virus, Theiler's encephalitis virus, Sendai virus, minute virus, mouse adenovirus, mouse hepatitis virus, lymphocytic choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus (assayed by M. A. Bioproducts, Walkersville, MD). The cultures were maintained for no longer than 12 weeks after recovery from frozen stocks.

Human umbilical vein endothelial cells were grown in Falcon "surface-modified", polystyrene flasks with complete Endothelial Cell Growth Medium. After adding the "SupplementMix" the concentrations of growth factors in the complete medium were as follows:

Fetal Calf Serum	*2%*
ECGS/H	*0.4%*
Epidermal Growth Factor	*0.1 ng/ml*
Hydrocortison	*1 µg/ml*
Basic Fibroblast Factor	*1 ng/ml*

The cells cultured in Endothelial Cell Growth Medium were checked regarding their morphology, the adherence rate and the population doubling time. All experiments were performed with cells passaged three to seven times after their receipt from the supplier.

Growth medium was changed every 2-3 days. The cells were split into the new culture flasks when they reached 80-90% confluence. Old medium was removed and the cells were washed twice with sterile PBS buffer. Then 1 ml of trypsin-EDTA solution was added, the culture flask was incubated at 37°C and observed under the microscope until cells detached from the surface of the flask. Then 10 ml of complete fresh medium was added to inactivate the activity of trypsin. Cells were centrifuged (except for HUVE cells) at 1000 g for 4 min at room temperature. Medium was discarded and the cells were resuspended in fresh growth medium.

5.2.1.2 Cell quantification and evaluation of viability

An efficient way of counting cells and at the same time the evaluation of percentage of viable cells is the technique of "dye exclusion". This test is based on the concept that viable cells do not take up some dyes, whereas dead cells are permeable to these dyes. Trypan blue is the most commonly used dye. In the cell culture some misleading situations such as recent trypsinization and freezing and thawing in presence of dymethylsulphoxide (DMSO) may lead to membrane leakeness. From each suspension cells an aliquot of 10 µl was harvested, mixed with 10 µl of tryplan blue and counted on a counting chamber under the microscope. The mean of at least three counts of viable cells (not stained with trypan blue)/quadrant was considered and multiplied to the magnitude (10^4) and the dilution factor.

5.2.1.3 Cell stimulation with different factors

For cytokine induction HUVE cells were maintained overnight (16 h) in the appropriate medium with 0.5% fetal calf serum (reduced medium) in order to synchronize the cell culture. The factors were diluted to the stock concentrations recommended by the manufacture in BSA 0.1% in sterile PBS. For further use the cytokines were diluted directly in the cell culture reduced medium at the indicated concentrations for desired period of time.

5.2.1.4 Storage of cells

In order to minimize the cellular injury induced by freezing and thawing procedures (intracellular ice crystals and osmotic effects), a cryoprotective agent DMSO was added. A variable number of L3.6pl cells (between 1-2 x 10^6) was spin down and resuspended in 10% DMSO solution (DMSO diluted in FBS). Afterwards, 1 ml aliquots of cell suspension were

dispensed into criotubes (1.8 ml). The tubes were placed into wells of a brass block pre-cooled at 4°C. The block was then kept at –70°C for 24 h after which the ampoules of cells were transferred to liquid nitrogen for long-term storage.

For revival of cells a frozen ampoule was thawed rapidly in a 37°C water bath, disinfected and the content was put in a cell culture flask with pre-warmed medium. After 6 hours the medium was discarded and fresh pre-warmed medium was added.

5.2.1.5 Detection of viable cells

Measurement of cell viability forms the basis for *in vitro* assays of a cell population's response to external factors. The reduction of tetrazolium salts is now widely accepted as a reliable way to examine cell proliferation. The yellow tetrazolium MTT (3-[4, 5-dimethylthiazolyl-2]-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means.

For an assessment of AZM475271 activity directed against cultured L3.6pl and HUVE cells, we used an *in vitro* TACS MTT Cell Proliferation and Viability Assay Kit. Cells were removed from subconfluent cultures by treatment with trypsin-EDTA. Trypsinization was stopped with complete medium. Cell suspension was harvested by centrifugation (400 g for 4 min at room temperature), 15×10^3 cells/well were plated in 96-well plates in complete medium (amount of the cells was determined by trypan blue method, 5.2.1.2). After 24 hours of attachment, cells were treated with AZM475271 (1 – 25 μmol/L), and the plates were incubated for another 72 hours (37°C, 5%CO_2). Cells were washed with PBS; the MTT Reagent was added according to the manufacturer's recommendations to each well, including controls. The plates were returned to cell culture incubator for 2 to 4 hours, and when the purple precipitate was clearly visible under the microscope the MTT Detergent was added to all wells, including controls. The plates were left with cover in the dark for 2 to 4 hours at room temperature. The absorbance in each well (OD, optical density) was measured at 570 nm in a microplate spectrophotometer. The IC_{50} (the concentration of AZM475271 at which 50% of cells were viable compared to cells grown in the absence of Src kinase inhibitor) was calculated using the following formula:

$$IC_{50} = OD \text{ of the cells treated / } OD \text{ of untreated cells x } 100\%$$

All experiments were replicated three times.

5.2.1.6 Inhibition of growth factor-mediated endothelial proliferation

HUVEC proliferation in the presence and absence of growth factors was evaluated using 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) incorporation. Briefly, HUVE cells were plated in 96-well plates (15×10^3 cells/well) in reduced medium and dosed with AZM475271 ± $VEGF_{165}$ or EGF (both growth factors at concentration 25 ng/ml were taken). The cultures were incubated for 72 hours (37°C, 5%CO_2) and then assayed for the incorporation of MTT using the microplate spectrophotometer, as described above (5.2.1.5). The IC_{50} dose of AZM475271 was assumed as the dose which inhibited 50% of HUVE cell proliferation.
All experiments were replicated at least three times.

5.2.1.7 Chemotaxis assay

Chemotaxis experiments were performed using BD BioCoat™ Matrigel invasion chambers. The apparatus consists of a BD Falcon TC companion plate with Falcon cell culture inserts containing an 8 μm pore size PET membrane with a thin layer of MATRIGEL Basement Membrane Matrix. The Matrigel Matrix serves as a reconstituted basement membrane *in vitro*. The layer occludes the pores of the membrane, blocking non-invasive cells from migration through the membrane. In contrast, invasive cells (malignant and non-malignant) are able to detach themselves from and invade through the Matrigel Matrix and the 8 μm membrane pores. The membrane may be processed for light and electron microscopy and can be easily removed after staining.
HUVECs (10^5 cells/well) resuspended in reduced medium were seeded into the upper well of the chamber system on a human fibronectin-coated polyethylene terephthalate membrane with 8 μm pores. Human recombinant $VEGF_{165}$ diluted in the cell culture reduced medium was added as a chemo-attractant into the lower well at 20 ng/ml. Inhibition of VEGF-induced chemotaxis was assessed after including AZM475271 at relevant doses to the upper compartment of the chamber. The cells were allowed to migrate for 4 hours at 37°C, after which the filter was fixed with cold methanol and stained with haematoxylin. The non-invading cells were removed from the upper surface of the membrane by "scrubbing" with a cotton swab, and the number of migrated cells was counted in 5 random 0.159-mm^2 fields at x100 magnification.
The average of triplicate inserts from three representative experiments was obtained.

5.2.2 Biochemical methods

5.2.2.1 Inhibition of protease/phosphatase activity

A commercially available cocktail of protease/phosphotase inhibitors (final concentrations: 0.02 mg/ml pancreas extract, 5 µg/ml pronase, 0.5 µg/ml thermolysin, 3 µg/ml chymotrypsin and 0.33 mg/ml papain) was prepared just before use and employed when needed.

5.2.2.2 Preparation of cellular extracts using RIPA buffer

During RIPA lysis intracellular and membrane proteins are solubilized due to the presence of detergent and high salt concentration in the lysis buffer. Nonsolubilized proteins are precipitated by centrifugation. Protease inhibitor cocktail is included in the lysis buffer to prevent proteolysis, phosphotase inhibitor cocktail - to maintain the phopsphorylation status of phosphoproteins, EDTA - to chelate divalent ions that are essential for metalloproteases.

Trypsinized HUVE cells (5.2.1.1) were washed twice with ice cold PBS, collected by centrifugation at 400 g for 4 min at 4°C. Cells were than resuspended in ice cold RIPA buffer supplemented with the cocktail of protease/phosphotase inhibitors to a final concentration of about 10^7-10^8 cells/ml. Cells were incubated on ice for 10 min and centrifuged at 14000 g at 4°C for 10 min. The supernatant containing total cellular proteins was collected and stored at –20°C. Total protein concentration in the supernatant was determined as described below (5.2.2.3).

5.2.2.3 Determination of protein concentration

The BCA™ Protein Assay Reagent Kit was used to measure protein concentration in cellular lysates. The BCA method combines the well-known reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium and selective colorimetric detection of the cuprous cation (Cu^{+1}) with a reagent containing bicinchoninic acid (BCA). The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous cation. This complex has a strong absorbance at 562 nm that is nearly linear with increasing protein concentrations.

Five µl of protein lysates were diluted in 45 µl of water and plated in 96-well plate. Protein standards for calibration were prepared by diluting 0, 5, 15 and 30 µg of bovine serum albumin (BSA) from 2 mg/ml stock solution in water to the final volume of 100 µl. Working BCA reagent was prepared freshly by mixing reagent A (containing bicinchoninic acid, sodium carbonate, sodium bicarbonate and sodium tartrate in 0.1M sodium hydroxide) and reagent B

(containing 4% cupric sulfate) from the kit at the ratio of 1:50 vol./vol.. Fifty µl/well of working BCA reagent was added to protein samples. Plates were placed in 60°C for 30 minutes, and then were allowed to cool down at room temperature for about 10 minutes. The absorbance was measured at or about 562 nm on the microplate spectrophotometer. A standard curve was prepared by plotting the average Blank-corrected 562 nm measurement for each BSA standard *vs.* its concentration in µg/ml. The standard curve was used to determine the protein concentration of each unknown sample.

5.2.2.4 Immunoprecipitation of proteins from cellular lysates

In this approach, specific antibody is added to the cellular lysate to bind protein of interest. Antibody-protein complexes are then precipitated using solid-phase matrix. Bacterial proteins A and G which have specific binding sites for Fc-parts of antibodies, covalently coupled to cross-linked agarose, are usually used as solid-phase matrix to precipitate protein-antibody complexes. Cellular lysates containing 0.5-1 mg of total protein were mixed with about 1 µg of antibody in pre-chilled 1.5 ml tubes on ice. Volumes of the mixture were adjusted to 200-300 µl to obtain equal protein concentrations in each sample. Tubes were incubated for 4 hours at 4°C with constant rotation. 20-25 µl of Protein A or G coupled agarose was added to each sample and tubes were incubated for additional 4 hours at 4°C with constant rotation. Following incubation with Protein A or G agarose, immuno-complexes were pelleted by centrifugation at 4500 g at 4°C for 1 minute and washed 3 times with ice-cold RIPA lysis buffer and one time with ice-cold 1X kinase buffer. Each time complexes were collected by centrifugation at 4500 g at 4°C for 1 minute. Five-10 µl of the kinase buffer used for the last wash were left above the agarose pellet. Ten µl of 2X Laemmli loading buffer were added to the samples and proteins were denatured by heating to 95°C for 5 minutes. Samples were cooled down on ice and analyzed immediately or frozen at –80°C for later analyses. Protein A or G agarose was pelleted by centrifugation at 14000 g at 4°C for 30 minutes and supernatants, containing immunoprecipitated proteins were analyzed by SDS-PAGE electrophoresis (5.2.2.5).

5.2.2.5 SDS-Polyacrylamidgelelectrophoresis PAGE

In this approach proteins in the mixture are denatured by heating in the presence of 2-mercaptoethanol and SDS. Denatured polypeptides bind SDS and become negatively charged. The amount of bound SDS is almost always proportional to the molecular weight of a

polypeptide, and is independent of its sequence. Therefore proteins and protein subunits are separated according to their size during migration through the pores in the gel matrix in response to an electrical field.

Protein samples for electrophoresis were prepared by dissolving a mixture of proteins in SDS-PAGE loading buffer and heating up the samples at 95°C for 5 minutes (5.2.2.4).

Separation gels with the following dimensions were used: thickness 1.0 mm, length 7.3 cm and width 8.3 cm. One µl TEMED per 1 ml of gel mix was used for the catalysis of the polymerization reaction, which was initiated by adding APS to a final concentration of 0.1%. The stacking gel was composed similar to the running gel, only Tris-HCl with pH 6.8 was used and the acrylamid had a final concentration of 3%. Proteins were separated at 250V and 30 mA until the dye front has left the separation gel.

5.2.2.6 Transfer of proteins to PVDF membrane

Proteins separated by SDS-PAGE were transfered to a PVDF membrane Hybond P. The transfer was done in a semi-dry TRANS-BLOT SD cell (Fig. 5.3) applying 25V and 100mA for 90 minutes. In semi-dry blotting the electrodes are placed directly in contact with the gel/nitrocellulose membrane sandwich to provide a fast, efficient transfer. Because of this direct contact there is a minimum of transfer buffer required for this process.

PVDF membrane was soaked in methanol for a few minutes and then transferred to a container with Towbin transfer buffer. Gel and attached PVDF membrane were sandwiched between two pieces of Whatman paper and soaked in the transfer buffer.

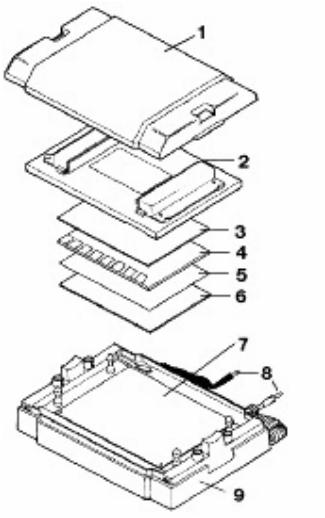


Figure 5.3 An exploded view of the Trans-Blot SD cell: 1, safety lid; 2, cathode assembly with latches; 3, filter paper; 4, gel; 5, membrane; 6, filter paper; 7, spring-loaded anode platform, mounted on four guide posts; 8, power cables; 9, base.

The prestained protein ladder served as a control for the transfer.

5.2.2.7 Ponceau S staining of proteins on PVDF membrane

Staining with Ponceau S was used to provide visiual evidence that electrophoretic transfer of proteins has taken place. Ponceau S is a negative stain which binds to the positively charged amino groups of the protein.

PVDF membrane was incubated for 5-10 seconds in Ponceau S staining solution and then washed with water until the protein bands became visible.

5.2.2.8 Analysis of proteins on PVDF membrane by immunoblotting

In this method, specific antibodies are used to identify proteins transferred to PVDF membrane. First, membrane is immersed in blocking buffer to fill all protein binding sites with non-reactive protein. Then membrane is incubated in a solution containing antibody directed against the antigen(s) in the protein to be detected. Primary antibody bound to the protein of interest are recognized by secondary antobody conjugated with horseradish peroxidase (HRP). The complex containing the antigen, primary antibody and secondary antibody-HRP conjugate is detected by chem luminescent visualization using ECL detection system.

PVDF membranes were blocked in the blocking solution either for 1-3 hours at room temperature or overnight at 4°C. After blocking, membranes were then washed once for 15 minutes and twice for 5 minutes in TBS-T buffer with agitation. After the washes membranes were incubated with antibodies appropriately diluted in 5% BSA in TBS-T overnight at 4°C with agitation. After incubation with primary antibodies, membranes were washed again as described above, then transferred to the containers with secondary antibody-HRP conjugates in 5% BSA in TBS-T and incubated for 1 hour at room temperature shaking gently. Membranes were then rinsed twice and washed with agitation twice for 15 minutes, twice for 10 minutes, and twice for 5 minutes. For detection, membranes were incubated in a mixture of ECL Western Blotting detection reagents for 2 minutes. Remaining drops of ECL buffer were removed and membranes were placed into a transparent folder. Hyperfilm ECL was then exposed for an appropriate time and developed with an AGFA developing system.

5.2.2.9 Stripping and re-probing of western blot

PVDF membranes were stripped and re-probed using Restore™ Western Blot Stripping Buffer. Twenty ml of Restore™ Western Blot Stripping Buffer were warmed to room temperature. Membranes were placed in the buffer to be stripped and incubated for 15 minutes at room temperature. After incubation, the blots were removed from the buffer and washed 3 times for 15 min in TBS-T. After determining that the membranes were properly stripped, next immunoprobing experiments were performed.

5.2.3 Determination of apoptotic cells by FACS analysis *via* propidium iodide staining

Propidium Iodide (PI) binds to double-stranded DNA, but it can only cross the plasma membrane of non-viable cells. For analysis by flow cytometry, the PI staining can be monitored in the FL2 channel.
HUVE cells were plated into T75 flasks and treated with different concentrations of AZM475271 (0 – 25 μM). After 12 hours, cells were collected and suspended in a Nicoletti buffer and incubated for 15 min protected from light at room temperature. The supernatant was discarded by centrifugation of cell suspension at 1300 g for 4 min. After 2 washing steps with PBS, cells were finally resuspended in sterile PBS and the DNA content present in the resulting nuclei was determined on a fluorescence-activated cell sorter (FACS). Signal height, area, and width were recorded for the PI channel. Data analysis was done using the WinMDI 2.8 software. Sub-G_0/G_1 was then quantified and used as an estimate of the amount of the cells undergoing apoptosis. The cellular debris was excluded from the analysis. Experiments were repeated three times.

5.2.4 Enzyme-linked immunoassays

5.2.4.1 Determination of human vascular endothelial growth factor concentrations in cell culture supernates

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for VEGF has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any VEGF present is bound by the immobilized antibody. After

washing away any unbound substances, an enzyme-linked polyclonal antibody specific for VEGF is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of VEGF bound in the initial step. The color development is stopped and the intensity of the color is measured.

The level of VEGF was measured in cell culture supernates collected on day 0, 2, and 4 from L3.6pl or HUVE cells following treatment with Src kinase inhibitor AZM475271 at different concentrations (0.1, 1 μM). The assay was performed according to the manufacturer's recommendations. Absorbance at 450 nm was measured and corrected using the 540-nm reading on the microplate reader. VEGF content was calculated according to the parameter of the calibration curve. Calibration curves with a correlation coefficient at least 0.998 were used. All experiments were replicated three times.

5.2.4.2 *In vitro* Src kinase inhibition test

A poly (Glu, Tyr) 4:1 random copolymer was used as the tyrosine-containing substrate. This is stored as a 10 mg/ml stock solution in PBS at 20°C and diluted 1:200 with PBS to coat 96-well plates (100 μl/well). Substrate was plated the day before the assay, and the plates were covered with adhesive seals and stored overnight at 4°C. On the day of the assay, the substrate solution was discarded, and the plates were then incubated with 120 μl/well of 5% BSA in PBS for 10 minutes. The plates were then washed once with PBS-T and incubated with 50 mmol/L HEPES (pH 7.4) at 100 μl/well until the next stage. Confluent HUVE or L3.6pl cells were treated with different concentrations of AZM475271 or PP2. After 12 hours cells were washed and lysed in 1 ml of ice-cold RIPA lysis buffer. Lysates were clarified by centrifugation at 14000 g for 10 minutes and active src was then precipitated using 15μg of v-src (Ab-1) monoclonal antibody pre-adsorbed to 50μl of protein G-sepharose. The immune complexes containing precipitated src were washed 3 times with ice-cold RIPA lysis buffer, one time with ice-cold 1X kinase buffer and used in kinase reaction as an active src kinase. Solutions of 80 μmol/L ATP in 80 mmol/L $MgCl_2$ and 80 mmol/L $MgCl_2$ alone (negative controls) were prepared. The HEPES was discarded from the substrate plates and the following additions were now made in this order: 25 μl/well ATP/$MgCl_2$ or $MgCl_2$ alone; 50 μl/well beads to start the reaction. The reaction time allowed was 30 minutes at room temperature on a plate shaker. The assay was stopped by washing the plates four times with PBS-T (150 μl/well). Detection of the resultant tyrosine phosphorylation was facilitated by the addition of an anti–phospho-tyrosine monoclonal antibody

conjugated to HRP (diluted 1:5000 in PBS-T + 0.5% BSA + 0.1 mmol/L sodium orthovanadate), added at 100 µl/well and incubated for 1 hour. The plates were again washed (six times). TMB substrate solution (100 µl/well) was added. After 5 minutes of color development, the reaction was stopped by the addition of 50 µl/well 0.8 mol/L H_2SO_4. Control and blank wells were included on all plates containing compound diluent and $MgCl_2$ solution with and without ATP, respectively, to determine the dynamic range of the assay. The *in vitro* VEGF-R2 kinase inhibition assay determines the ability of AZM475271 to inhibit VEGF-R2 kinase activity and has been used as a selectivity screen. The method was performed as reported previously (Lu et al., 2003).

5.2.5 *In vitro* study of angiogenesis

5.2.5.1 Aortic ring assay

Angiogenesis was studied by culturing aortic explants in three-dimentional matrix gels according to the procedure of Nikosia and Ottineri (Nikosia et al., 1990). Thoracic aortas were removed from 6- to 8- week-old male ACI rats and immediately transferred to a culture dish containing cold serum-free Dulbecco´s Minimal Essential Medium. The peri-aortic tissue was carefully removed with fine microdissecting forceps and scissors, paying special attention not to damage the aortic wall. One mm long aortic slices (approximately 15 per one aorta) were sectioned and extensively rinsed in 5 consecutive washing steps with D-MEM. Ring-shaped explants of the aorta were then embedded on Matrigel-coated 24-well plates. HEPES-buffered D-MEM containing AZM475271 in different concentrations was added and the plates were incubated at 37°C, 5% CO_2, for 4 days. Fresh medium with respective additives was reintroduced into the cultures on day 2. The rings were examined by phase contrast microscopy with a Zeiss Axiotech Vario microscope at ×10 magnification. An estimation of the length of the capillary was performed by measuring the distance from the aortic explant to the approximate mean point of capillary. The length of the capillary was measured using AxioVision software.

5.2.5.2 Spheroid angiogenesis assay

To evaluate the anti-angiogenic properties of AZM475271, we used an *in vitro* angiogenesis assay as described in detail, previously (Korff et al., 1998). 10^3 HUVECs (passage 4 to 6) per spheroid were plated into a non-adhesive, round bottom 96-well plate. After 24 hours, the

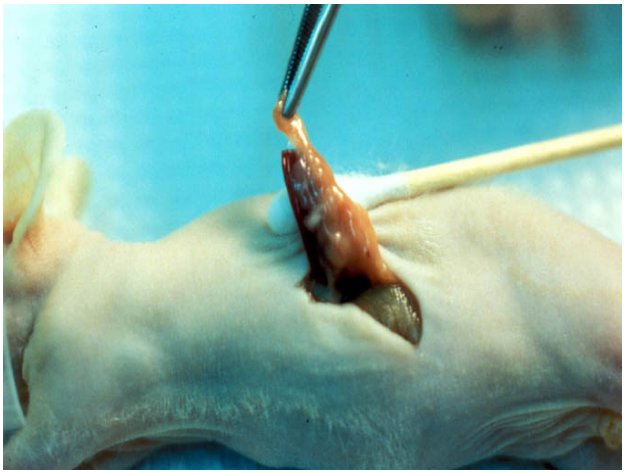
spheroids were harvested and half the spheroids of a 96-well plate (approximately 48 spheroids) were embedded in 1 ml of a collagen matrix and transferred into a 24-well plate. For the next 24 hours we incubated the embedded spheroids with the allocated treatment and thereafter, the spheroids were fixed in a 4% formalin solution to prepare them for the subsequent analysis. Spheroid sprouting was stimulated with human recombinant VEGF-A_{165} (25 ng/ml) in the presence or absence of AZM475271. The pictures of the spheroids were taken under trans-illumination using an Achroplan objective (n.a. = 0.45) providing a 20-fold magnification.

5.2.6 Orthotopic xenograft pancreatic tumor model

5.2.6.1 Tumor cell implantation

For *in vivo* injection, L3.6pl human pancreatic carcinoma cells were harvested from culture flasks by a treatment with trypsin-EDTA and resuspended in sterile ice-cold PBS. Only single-cell suspensions of >90% viability (trypan blue exclusion) were used for injection. Male nude mice were anesthetized with ketavet (100 mg/kg mouse body weight) and xylazin (5 mg/kg mouse body weight) followed premedication with atropine sulfate. A small left abdominal flank incision was made and the spleen exteriorized (Fig. 5.4a). L3.6pl tumor cells ($1x10^6$ in 40µl PBS) were injected subcapsularly in a region of the pancreas just beneath the spleen. We used a 30-gauge needle, a 1 ml disposable syringe, and a calibrated pushbutton-controlled dispensing device to inject the tumor cell suspension (Fig. 5.4b). A successful subcapsular intrapancreatic injection of tumor cells was identified by the appearance of a fluid bleb without intraperitoneal leakage (Fig. 5.4c). To prevent such leakage, a cotton swab was held for 1 min over the site of injection. One layer of the abdominal wound was closed by suture.

A



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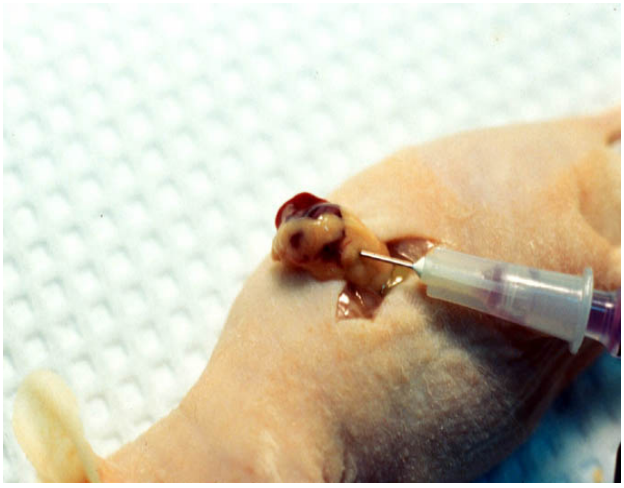


C

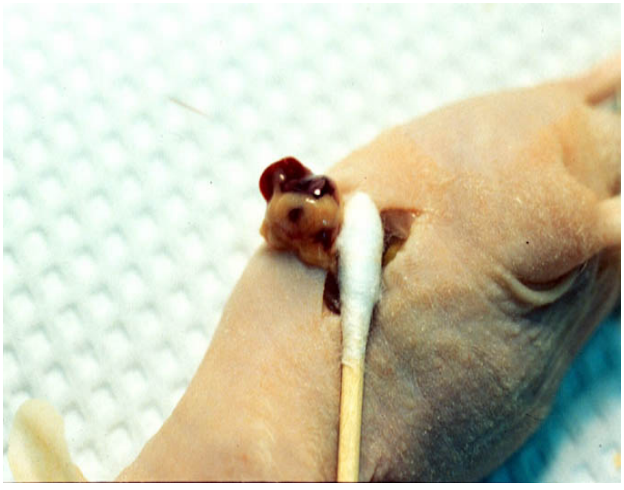


Figure 5.4 Orthotopic tumor cell injection

The animals tolerated the surgical procedure well, and no anesthesia-related deaths occurred.

5.2.6.2 Treatment of established tumors growing in the pancreas of nude mice

Seven days after implantation of tumor cells, mice were randomly assigned to one of the following groups of 5-9 mice each:

1) Daily intragastral administration of Src tyrosine kinase inhibitor AZM475271 at a dose of 25 mg/kg mouse body weight;
2) Daily intragastral vehicle solution for AZM475271 (Tween 20 diluted 1:100 in NaCl).

5.2.6.3 *In vivo* evaluation of plasma concentration levels of AZM475271

Healthy nude mice were treated with a single dose of 50 mg/kg AZM475271 by oral administration. The plasma concentration of AZM475271 was measured by mass spectrometry 2, 6, and 24 hours after oral feeding of the compound.

5.2.6.4 Monitoring of mouse body weight and tumor volume

Starting 3 days after the initiation of therapy with AZM475271, the measurement of tumor volume (by transcutaneous palpation) and mouse body weight was performed.

5.2.6.5 Necropsy procedure and histopathological studies

The animals were sacrificed 32 days after the initiation of treatment, when > 50% of the control animals had become moribund. Primary pancreatic tumor size, liver and lymph node metastasis, and local peritoneal carcinosis were assessed. All palpable or visible masses in the pancreas were considered pancreatic tumors and the presence of tumor was later confirmed by Haematoxylin and Eosin (H&E) staining. Excised pancreatic tumors were weighed. The tumor volume was then calculated using the formula $V = {}^{\pi}/_{6}\,(a \times b \times c)$, where a, b and c represent the length, width, and height of the mass. Microscopically, tumor nodules ≥1 mm in diameter were counted on the entire liver surface. Where visible liver metastases were evident, the tissue was processed for H&E staining to confirm the macroscopic observations. Furthermore, macroscopically-enlarged regional lymph nodes (celiac and para-aortic) were excised and H&E staining performed to

confirm the presence of metastases. Tumor lesions were harvested, some were fixed in 10% buffered formalin and embedded in paraffin, and some were embedded in optimum cutting temperature (OCT) compound, snap-frozen in liquid nitrogen, and stored at -70°C.

5.2.7 Immunohistochemical analyses

5.2.7.1 Immunohistochemistry of paraffin embedded tissues

5.2.7.1.1 Haematoxylin and Eosin staining

Staining of the nucleus of the cells was done with haematoxylin; eosin was used for the cytoplasm staining. Samples were deparaffinized by incubation in xylene for 20 min, 2 minutes in 100%, one minute in 96 %, one minute in 75% ethanol and finally washed in distilled water. The samples were then rinsed in haematoxylin for one minute, washed in distilled water and incubated with eosin for 2 minutes. After washing the slides were mounted with Mayer gel.

5.2.7.1.2 Staining for Ki-67 antigen (The assessment of cell proliferation)

Evaluation of cell proliferation was performed using *Ki*-67-specific polyclonal rabbit anti-human antibody. Tissues were embedded in paraffin and 4–6 μm sections were prepared and mounted on positively-charged superfrost slides. Sections were dried overnight, deparaffinized in xylene and incubated in 100%, 95% and 80% ethanol (v/v in distilled water), before rehydrating in PBS. *Ki-67* antigen retrieval was achieved by microwaving tissue sections for 15 minutes at 750W. After cooling down, the slides were rinsed with PBS, and nonspecific binding sites were blocked with 5% bovine serum albumin (BSA) in PBS. After another washing step with PBS, the primary antibody (1:75, a polyclonal rabbit anti-human antibody against *Ki-67)* was applied, and the slides were incubated for 2 hours at room temperature. The samples were then incubated with biotinylated goat anti-rabbit secondary antibody (1:200) for 1 hour at room temperature, followed by incubation with an avidin-biotinylated horseradish peroxidase (HRP) complex from an ABC kit. Sections were examined microscopically and the average number of cells staining positively for *Ki-67* per high-power field (0.159 mm^2) was counted at x100 magnification.

5.2.7.2 Immunohistochemistry of snap-frozen tissues

5.2.7.2.1 Terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) staining

Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) staining was performed using a commercially available apoptosis detection kit (DeadEnd™ Fluorometric TUNEL System). In many cell types, apoptosis is characterized by the generation of DNA fragments through the action of endogenous endonucleases. The DNA of apoptotic cells is cleaved into multimers of 180–200bp fragments, corresponding to the oligonucleosomal size. Therefore, the DNA of apoptotic cells typically migrates as a ladder of 180–200bp multimers on an agarose gel. The generation of single strand breaks also has been reported. The DeadEnd™ Fluorometric TUNEL System measures the fragmented DNA of apoptotic cells by catalytically incorporating fluorescein-12-dUTP(a) at 3´-OH DNA ends using the enzyme Terminal Deoxynucleotidyl Transferase (TdT). TdT forms a polymeric tail using the principle of the TUNEL (TdT-mediated dUTP Nick-End Labeling) assay. The fluorescein-12-dUTP-labeled DNA can then either be visualized directly by fluorescence microscopy or quantitated by flow cytometry.

Tissue sections (8-10 µm thick) were fixed by immersing slides in freshly prepared 4% methanol-free formaldehyde solution in PBS (pH 7.4) in a Coplin jar for 25 minutes at 4°C and washed twice by immersing in fresh PBS for 5 minutes at room temperature. Then cells were premeabilized by immersing the slides in 0.2% Triton X-100 solution in PBS for 5 minutes and then rinsed twice in PBS for 5 minutes. After removing excess liquid by tapping, slides were covered with 100µl of equlibration buffer and equilibrated at room temperature for 10 minutes. Then, a sufficient TdT incubation buffer for all experimental and optional positive control reactions was prepared according to manufacture protocol. 50µl of TdT incubation buffer were added, slides were covered with plastic coverslips to ensure even distribution of the reagent and incubated at 37°C for 60 minutes inside the humidified chamber covered with aluminium foil to protect from direct light. After the incubation plastic coverslips were removed and reaction was terminated by immersing the slides in 2X SSC in a Coplin jar for 15 minutes at room temperature. Samples were washed twice by immersing the slides in PBS for 5 minutes at room temperature to remove unincorporated fluorescein-12-dUTP. Slides were mounted using glass coverslips and DEPEX gel. Samples were immediately analysed under a fluorescence microscope using a standard fluorescent filter set to view the green fluorescence of fluorescein at

520 ± 20nm. Results were expressed as the average of apoptotic cells in 10 random fields at x40 magnification.

5.2.7.2.2 Staining for CD31

Frozen tissue sections (8-10 μm thick) were fixed with cold acetone for 5 min, acetone and chloroform (1:1, v/v) for 5 min, and acetone for 5 min, then washed in PBS. Endogenous peroxidase was blocked by incubation of slides in 3% H_2O_2 diluted in methanol for 5 min and 3x3 min washed in PBS. Non-specific binding sites were blocked for 20 min with 5% horse serum and 1% goat serum in PBS. After the incubation, primary antibodies dissolved in 1% blocking buffer were applied and incubated overnight at 4°C: CD-31, monoclonal rat anti-mouse (1:200) which reacts to a surface antigen (CD-31) presented on all endothelial cells. Next day the samples were washed 3x3min in PBS and the slides were incubated for 10 min with blocking buffer at room temperature. Then secondary antibodies (biotinylated polyclonal rabbit anti-rat immunoglobulin, Ig-fraction, HRP- conjugated (1: 400) dissolved in blocking buffer were applied for 1 hour at room temperature. After washing 3x3 min in PBS the slides were rinsed with 0.2% Tween solution in PBS. Then the samples were incubated with AEC chromogen dissolved in H_2O_2 substrate, washed in distillate water and stained with haematoxylin for 1 min. After washing with distillate water the slides were mounted using glass cover slips and DEPEX gel. For the quantification of microvascular density, 10 random 0.159-mm^2 fields at x100 were captured for each tumor using an AxioCam camera mounted on a Carl Zeiss universal microscope and AxioVision software. Two investigators counted the microvessels independently in a blinded fashion. Tissues were examined at a low power (x40), and the three x200 fields of highest microvessel density were identified for vessel counts. Microvessels were quantitated according to the method described by Bruns (Bruns et al., 2000). Clusters of stained endothelial cells distinct from adjacent microvessels, tumor cells, or other stromal cells were counted as one microvessel. The results were expressed as the highest number of microvessels identified within a single x200 field.

5.2.7.2.3 Immunofluorescence double staining for CD31 and TUNEL

Frozen tissue was used for CD31/TUNEL immunofluorescence double staining. The procedure used is as described previously (Baker et al., 2002).

TUNEL assay was done with the use of a commercial apoptosis detection kit as described above (5.2.7.2.1). TUNEL-positive apoptotic cells were detected by localized green fluorescence within the cell nuclei, and endothelial cells were identified by red fluorescence. Apoptotic endothelial cells were identified by yellow fluorescence within the nuclei. The total number of apoptotic cells was quantified in 10 randomly selected microscopic fields and expressed as the ratio of apoptotic endothelial cells to the total number of endothelial cells in 5–10 random 0.011-mm^2 (at x400 magnification).

5.2.8 Statistical analysis

Pancreatic tumor weight, mouse body weight, and quantification of Ki67, TUNEL, and CD31 were compared using one-way ANOVA with a Student-Newman-Keuls multiple comparison test. The relative rates of liver and lymph node metastases within groups were compared by Fisher´s exact test. Survival analysis was computed by the Kaplan–Meier method and compared by the log-rank test (Hosmer et al., 1980). Significance was taken as $p < 0.05$. Results of the ELISA tests, proliferation assays, migration and aortic ring assays were analyzed using the paired Student's t-test with $p < 0.05$ considered to be significant.

Chapter 6

RESULTS

The initial goal of the work described in this thesis was to demonstrate the *in vivo* efficacy of the Src tyrosine kinase inhibitor AZM475271 in an orthotopic xenograft pancreatic tumor model. However, early results, which have raised questions as to the role of Src family kinases in tumor angiogenesis and metastasis, lead to a change the direction of the project.

6.1 *In vitro* enzyme inhibition in HUVE and L3.6pl cells

AZM475271 is a potent inhibitor of the Src tyrosine kinase activity in HUVE and L3.6pl cells. To demonstrate the selectivity of AZM475271, an *in vitro* Src inhibition ELISA was performed measuring the IC_{50} of AZM475271 necessary to prevent the ability (*i.e.* activity) of src kinase precipitated from HUVE or L3.6pl cells to phosphorylate an immobilized substrate in the presence of ATP. AZM475271 demonstrated a strong inhibition of Src kinase activity in HUVE and L3.6pl cells in a dose-dependent manner. The IC_{50} concentration of AZM475271 to inhibit the phosphorylation of Src in HUVE and L3.6pl cells was 0.1 µmol/L (Fig. 6.1, a and b, respectively). 4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine (PP2) was used as a positive control for Src kinase inhibition.

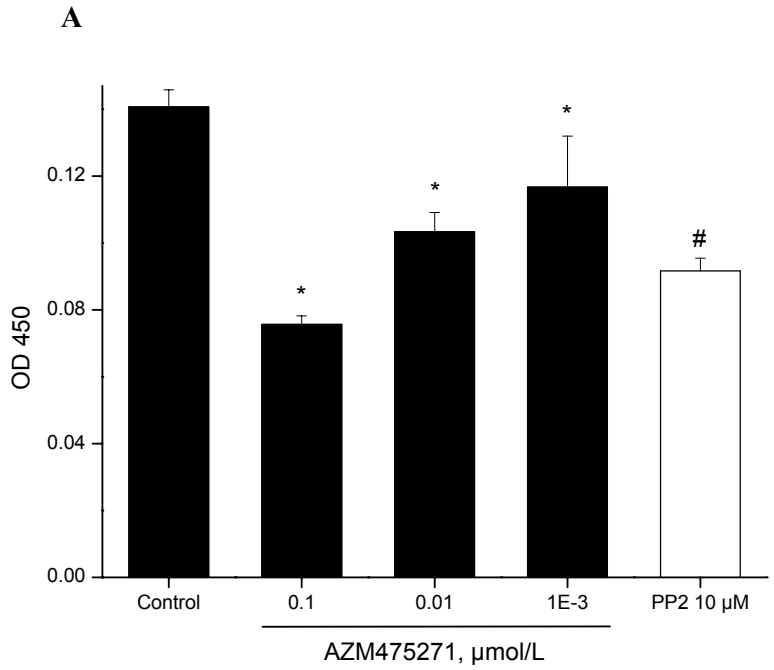
6.2 *In vitro* selectivity profile of AZM475271

A receptor tyrosine kinase inhibitor should prevent the phosphorylation of tyrosine residues on protein substrates following stimulation of the kinase with its specific ligand. The ability of AZM475271 to inhibit receptor tyrosine kinase activity (at the Michaelis constant [Km] for adenosine triphosphate [ATP]) was examined using an ELISA with recombinant cytoplasmic domains of Src non-receptor tyrosine kinases. Table 6.1 presents the kinase inhibition and selectivity profile of AZM475271 (data were kindly provided by AstraZeneca Pharmaceuticals). AZM475271 has considerable selectivity for Src *versus* kinase insert domain-containing receptor (KDR) (IC_{50} = 20.9 µmol/L), fms-like TK 1 (Flt-1) (IC_{50} > 100 µmol/L), fibroblast growth factor (FGF) TK, fms-like TK 4 (Flt-4), aurora kinase (AUR-3), mitogen-activated protein kinase (MAPK) kinase (MEK) (IC_{50} > 10 µmol/L), cyclin-dependent kinase-2 (CDK-2) (IC_{50} > 9 µmol/L), epidermal growth factor receptor (EGF) TK (IC_{50} = 2.6 µmol/L). AZM475271 has equipotent activity against Src family members c-Yes and Lck ($IC_{50} \leq 0.004$ µmol/L). Since the kinase domain is virtually identical in each member, it would be reasonable to assume that AZM475271 will inhibit the other family members with similar potency. The activities of Src kinases are normally highly regulated and AZM475271 will inhibit those that have been

activated. AZM475271 has poor activity against Csk, a negative regulator of Src (IC_{50} = 0.84 µmol/L).

Kinase	**IC_{50} (µmol/L)**	**Fold selectivity *vs.* Src**
Src	0.0027	
KDR (kinase insert domain-containing receptor)	20.9	>7700
Csk (negative controller of Src)	0.843	312
Flt-1 (VEGFR1)	>100	>37000
Flt-4 (VEGFR3)	>10	>3700
FGF TK	>10	>3700
EGF TK	2.59	960
MEK	14.03	>5000
CDK-2	9.753	3612
c-Yes (ubiquitous Src family member)	0.004	1.48
Lck (T cell restricted Src family member)	<0.004	<1.48

Table 6.1 AZM475271 inhibition of Src non-receptor tyrosine kinase and selectivity profile.



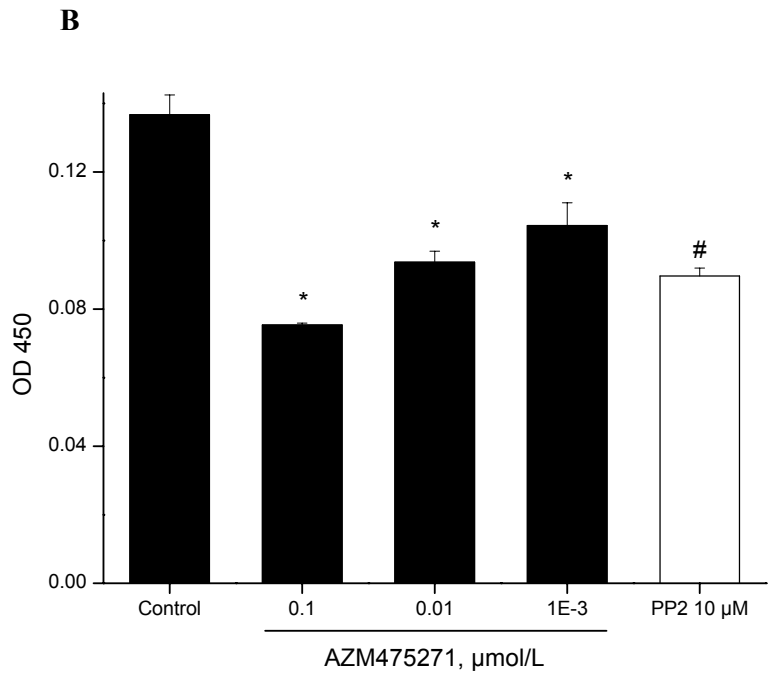


Figure 6.1 In vitro inhibition of Src Kinase.
ELISA was performed measuring the IC_{50} of AZM475271 necessary to inhibit the activity of src kinase, expressed in L3.6pl (A) or in HUVECs (B). 4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine (PP2) was used as a positive control for src kinase inhibition (*, $p<0.001$ *versus* control reaction; #, $p<0.001$ *versus* control reaction).

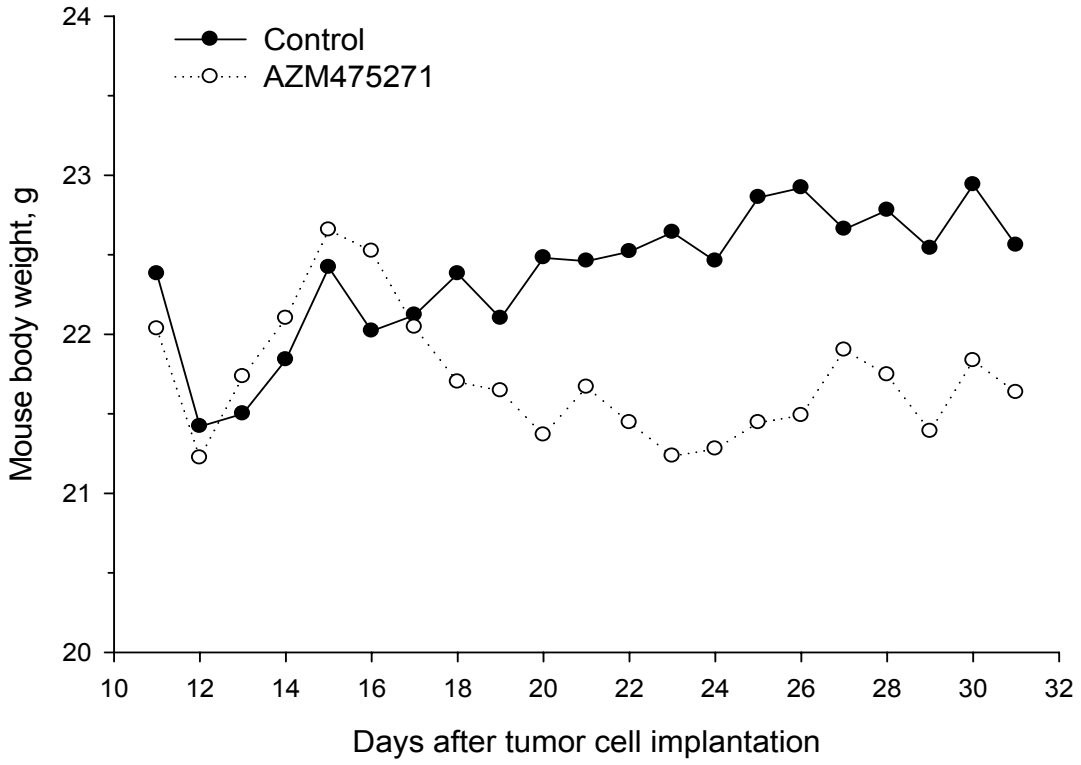
6.3 AZM475271 inhibits tumor growth and metastasis in an orthotopic nude mouse model

Tumors generated by orthotopic implantation of the metastatic L3.6pl cell line were used to evaluate the effects of AZM475271 on growth, metastasis, and angiogenesis in a nude mouse model. Pancreatic tumors were allowed to become established for 7 days before initiation of treatment. Starting 3 days after the initiation of therapy with AZM475271, the measurement of mouse body weight and tumor volume (by transcutaneous palpation) was performed (Fig. 6.2, a and b). Treatment with AZM475271 did not significantly change animal weight (at the end of experiment the mean animal body weight after therapy with AZM475271 was 21.6 g, compared to the untreated group with 22.6 g) (Fig. 6.2a). Monitoring of the tumor volume over the course of experiment revealed a decrease in the tumor growth progression in all animals treated with AZM475271 (Fig. 6.2b).

The animals were sacrificed 32 days after the initiation of treatment, when > 50% of the control animals had become moribund. At the time of necropsy, all control and treated mice had developed primary pancreatic tumors. Primary pancreatic tumor size, liver and lymph node metastasis, and local peritoneal carcinosis were assessed. Treatment with AZM475271 significantly reduced spontaneous liver metastasis (no animals had metastases) compared with treatment using vehicle solution. Mice who received AZM475271 had also a significant reduction in the incidence of lymph node metastases (five of nine animals) (Table 6.2). Tumor weight for treatment group was assessed using Fisher's Exact test. Other comparisons were made using the unpaired Student's t-test. The mean tumor volume was significantly decreased (~40% inhibition of primary tumor growth) in all animals treated with AZM475271 (AZM475271-treated animals, 817 mm^3; control animals, 1359 mm^3). Primary pancreatic tumor weight after AZM475271 therapy was also significantly less than that in control mice (mean weight 1.03 and 1.45, respectively). Results are presented in the Figures 6.3a and 6.3b.

In addition, daily intragastral administration of AZM475271 was well tolerated.

A



B

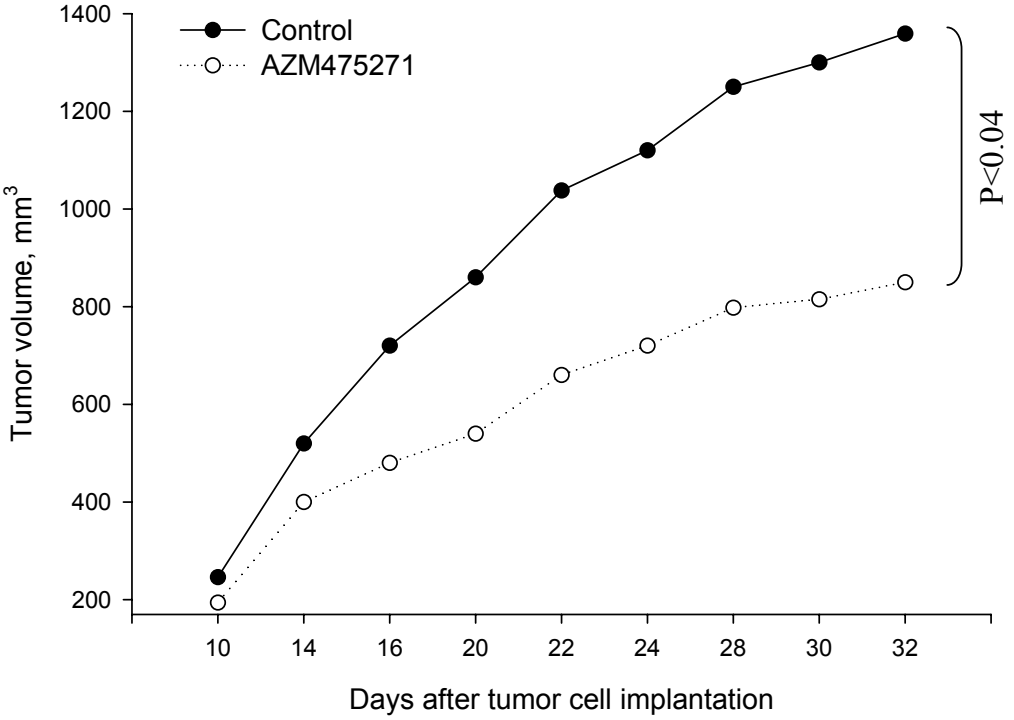
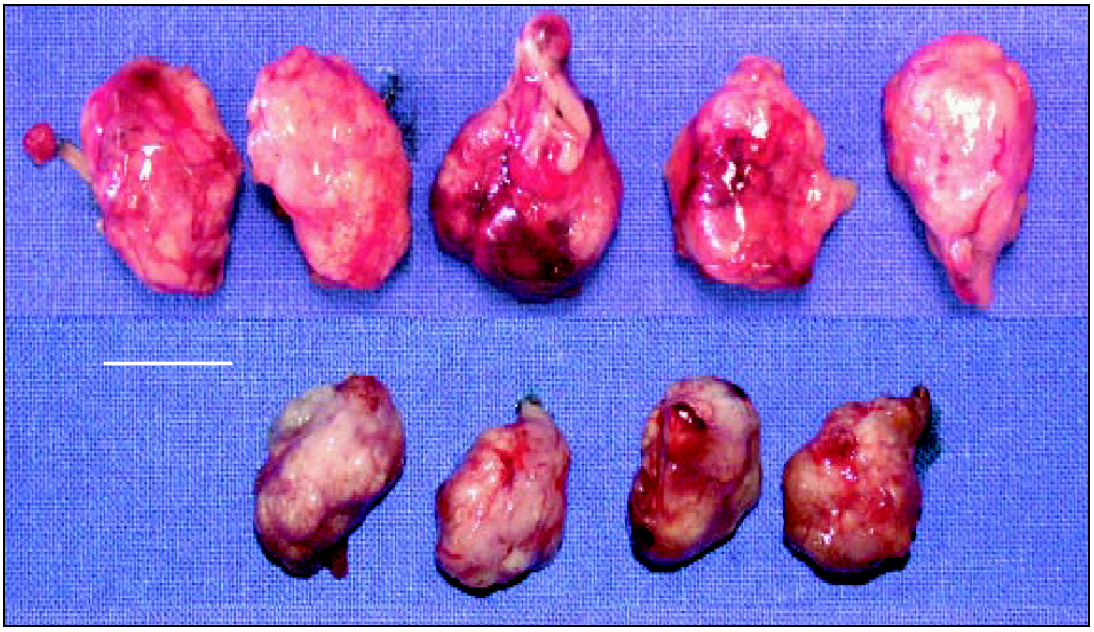


Figure 6.2 Monitoring of the mouse body weight (A) and L3.6pl tumor growth progression (B) in the orthotropic nude mouse model.

Treatment group	**Incidence of macroscopic tumors**[a]		
	Pancreas tumor	Liver metastasis[b]	Regional LN metastasis
Saline control	5/5	3/5	5/5
AZM475271	9/9	0/9[c]	5/9[c]

Table 6.2 In vivo therapeutic efficacy of AZM475271 on primary pancreatic tumor growth and metastasis in the orthotopic nude mouse model
[a] Incidence presented as number of animals with tumor type/ number of animals in group
[b] Visible nodules (≥1 mm in diameter)
[c] $p<0.03$ compared with control (Fisher's Exact Test)

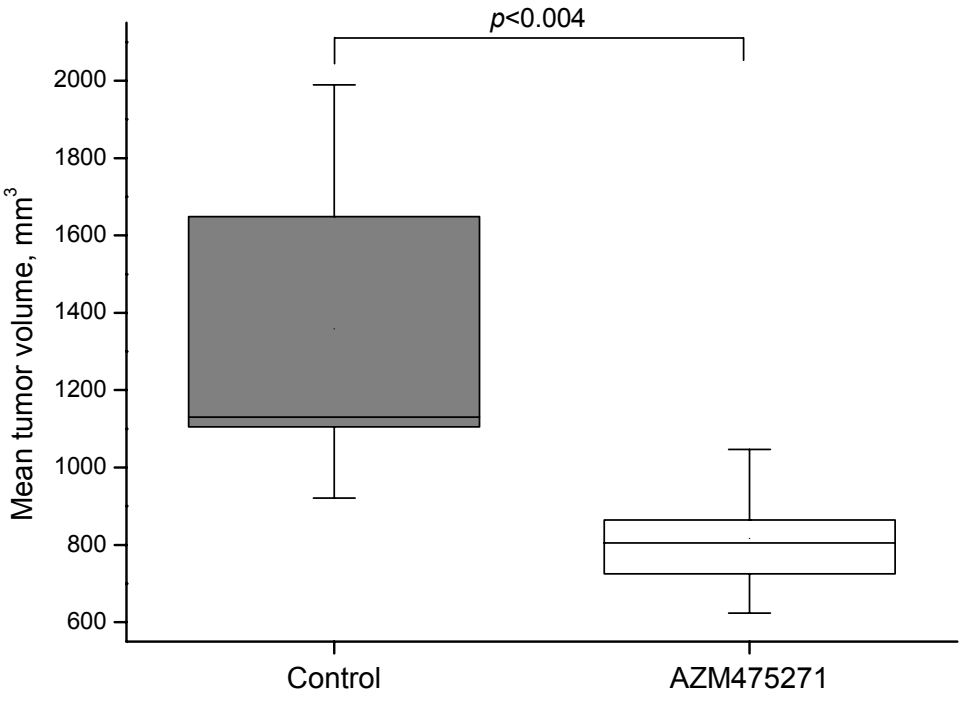
The photograph below shows the tumors harvested from the mice in the experiment on day 32. *Scale bar, 1 cm.*



Control

AZM475271

A



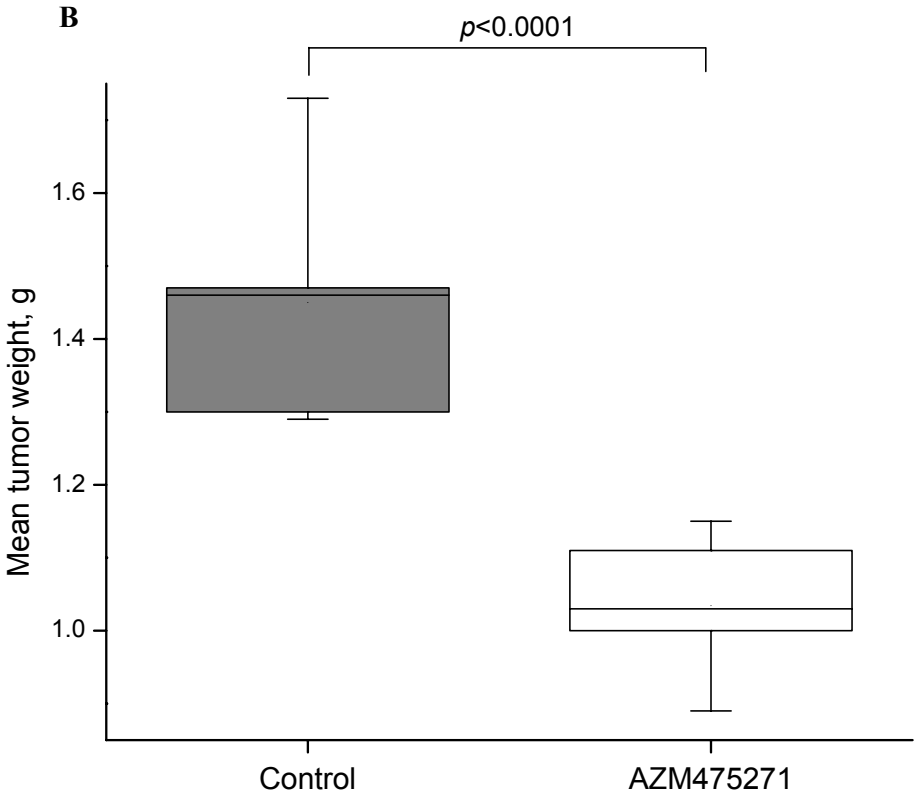


Figure 6.3 In vivo inhibition of L3.6pl pancreatic tumor volume (A) and pancreatic tumor weight (B) in the orthotopic nude mouse model.
L3.6pl human pancreatic cancer cells were injected into the pancreas of nude mice. Seven days after implantation of tumor cells, mice randomly were assigned to one of the following groups of 5-9 mice each: 1) daily intragastral administration of Src tyrosine kinase inhibitor AZM475271 at a dose of 25 mg/kg; 2) daily intragastral vehicle solution for AZM475271. The animals were sacrificed 32 days after the initiation of treatment.
Box plots represent the means for each group with standard deviation of data (*min* and *max*) and standard error of the mean.

6.4 *In vivo* evaluation of plasma concentration levels

After a single oral dose (gavage) of 50 mg/kg AZM475271 to healthy nude mice, the plasma concentration was measured 2, 6, and 24 hours later. The plasma concentration of AZM475271 in nude mice was 32.1 µmol/L (14.206 ng/ml), 20 µmol/L (8879 ng/ml), and 11.7 µmol/L (5187 ng/ml) at 2, 6, and 24 hours after oral dosing with 50 mg/kg AZM475271 (Figure 6.4).

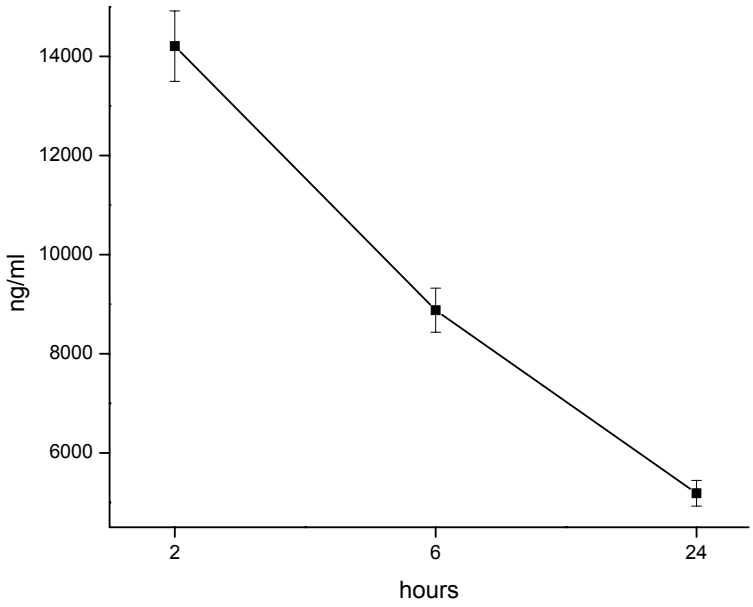


Figure 6.4 The plasma concentration of AZM475271 in nude mice after the single oral dose of 50 mg/kg AZM475271. Error bars are expressed as a 5% average of data.

6.5 Histological analysis of tissue sections

6.5.1 *In vivo* effect of AZM475271 on tumor cell proliferation

We next analyzed the effect of AZM475271 on tumor cell proliferation *in vivo* by assessing the level of the nuclear antigen *Ki-67*, which is present in all phases of the cell cycle except G_0. The mean number of *Ki-67*-positive tumor cells in the pancreatic tumors of control mice was 480 ± 14. After therapy with AZM475271, the mean number of *Ki-67*-positive cells was 185 ± 85 (reduced over more than 50 % compared to control mice) (Table 6.3; Fig. 6.5). These results indicate the *in vivo* antiproliferative effect of AZM475271.

6.5.2 *In vivo* effect of AZM475271 on tumor cell apoptosis

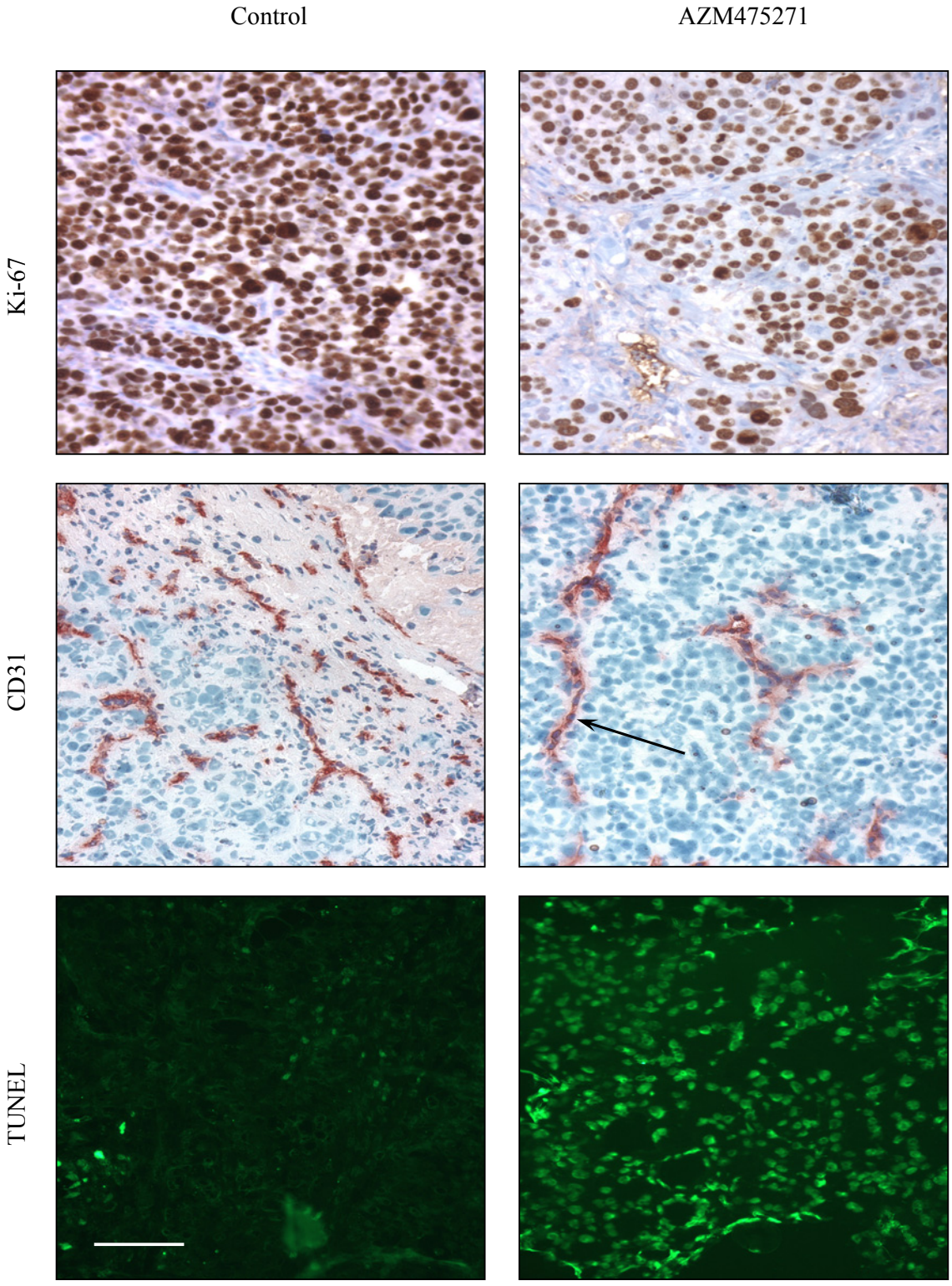
To determine the ability of AZM475271 to induce apoptosis in pancreatic carcinoma cells TUNEL staining was carried out. The mean number of TUNEL-positive cells was 8 ± 2 and 26 ± 11 ($p < 0.05$ *versus* control) in control and AZM475271-treated group, respectively. Results showed a significant enhancement of apoptotic tumor cells in the AZM475271 therapy group compared to control tumors. (Table 6.3; Fig. 6.5).

	Positively staining cells (mean ± SD)[a]	
	Tumor cells	
Treatment group	*Ki-67*	TUNEL
Saline control	480 ± 14	8 ± 2
AZM475271	185 ± 85[b]	26 ± 11[c]

[a] Positive cells per field determined from measurement of 10 random 0.159-mm^2 fields at ×100 magnification.
[b] $p<0.001$ compared with controls.
[c] $p<0.05$ compared with controls.

Table 6.3 Quantification of *Ki-67*- and TUNEL-positive cells in primary pancreatic tumors after therapy with AZM475271.

A



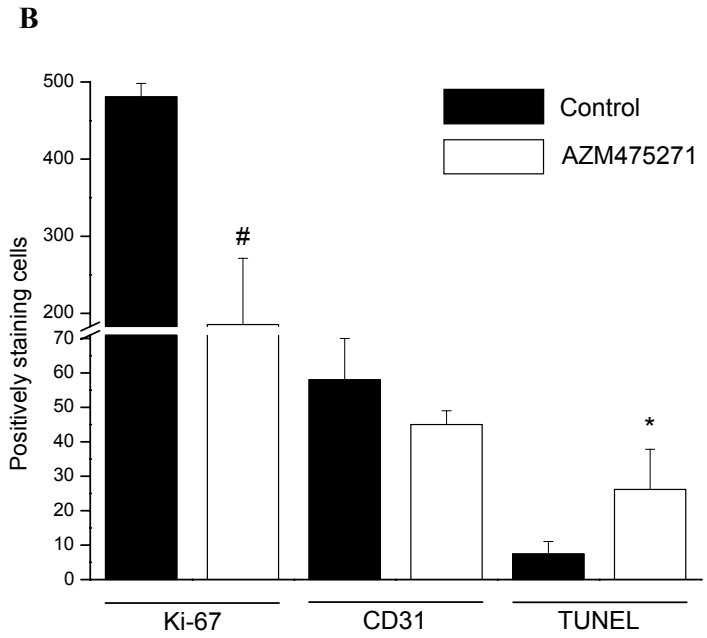


Figure 6.5 Immunohistochemical analyses of proliferation (*Ki-67*), microvessel density (CD31), and apoptotic cells (TUNEL) in L3.6pl pancreatic tumors growing in nude mice.

A, histological sections of control and AZM475271-treated tumors; *B*, mean number of positively stained cells per field (bars represent mean ± SD), $^{\#}$ $p<0.001$ compared with controls; * $p<0.05$ compared with controls.

Histopathologic evaluation of cell proliferation was performed using *Ki*-67 staining. Sections were examined microscopically and the average number of cells staining positive for *Ki-67* per high-power field (0.159 mm^2) was counted at ×100 magnification. The mean number of *Ki-67* positive tumor cells after therapy with AZM475271 was reduced by > 50 % compared with control mice. Frozen tissues were used for the evaluation of microvessel density (CD31-positive structures). CD31 staining demonstrated no significant influence of AZM475271 on MVD of tumors compared with controls ($p = 0.14967$, the arrow delineates CD31-positive cells).

In contrast, terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) staining for apoptotic cells revealed an increase in the number of cells undergoing apoptosis in pancreatic cancer in the AZM475271 group. The number of TUNEL-positive cells was counted in 10 random fields (0.159 mm^2) at ×100 magnification (bars = 100 μm).

6.5.3 Quantification of microvessel density in primary pancreatic tumors

Frozen tissues were used for the evaluation of microvessel density (CD31-positive structures). Clusters of stained endothelial cells distinct from adjacent microvessels, tumor cells, or other stromal cells were counted as one microvessel. The number of CD31-positive microvessels

counted per x100 field in the pancreatic tumors was lower in mice after therapy with AZM475271, but not significant (p = 0.14967) (Figure 6.5, the arrow delineates CD31-positive cells).

6.5.4 *In vivo* effect of AZM475271 on endothelial cell survival

We speculated that tumor cell apoptosis may have been due to poor perfusion after endothelial cell apoptosis rather then due to decreasing in microvessel density. Therefore, we developed a technique with which we could evaluate endothelial cell apoptosis *in vivo* in tissue sections. Endothelial cells were detected by localized red fluorescence, whereas green fluorescence was detected within the nuclei of apoptotic cells. Double-labeling of endothelial cells undergoing apoptosis resulted in localized yellow fluorescence. The total number of apoptotic cells was quantified in 10 randomly selected microscopic fields and expressed as the ratio of apoptotic endothelial cells to the total number of endothelial cells in 5–10 random 0.011-mm^2 (at x400 magnification).

The number of endothelial cells undergoing apoptosis was significantly greater in pancreatic tumors from mice treated with AZM475271 compared to control tumors (24 ± 9.1 for AZM475271 compared with 0.7 ± 1.5 for untreated control, p = 0.01) (Fig. 6.7). Clusters of apoptotic tumor cells were seen surrounding apoptotic endothelial cells when treated with AZM475271 (Fig. 6.6, the arrows delineate TUNEL-positive endothelial cells).

This finding suggests that the reduced tumor size that followed therapy with Src inhibitor was not wholly a direct consequence of inhibition of tumor cell proliferation. There was a statistically significant induction of endothelial cell apoptosis in the pancreatic tumor model when AZM475271-treated tumors and control tumors were compared, suggesting that the reduction in tumor growth and metastasis in the treated group was due to endothelial cell death and not to a direct effect on the tumor cells themselves.

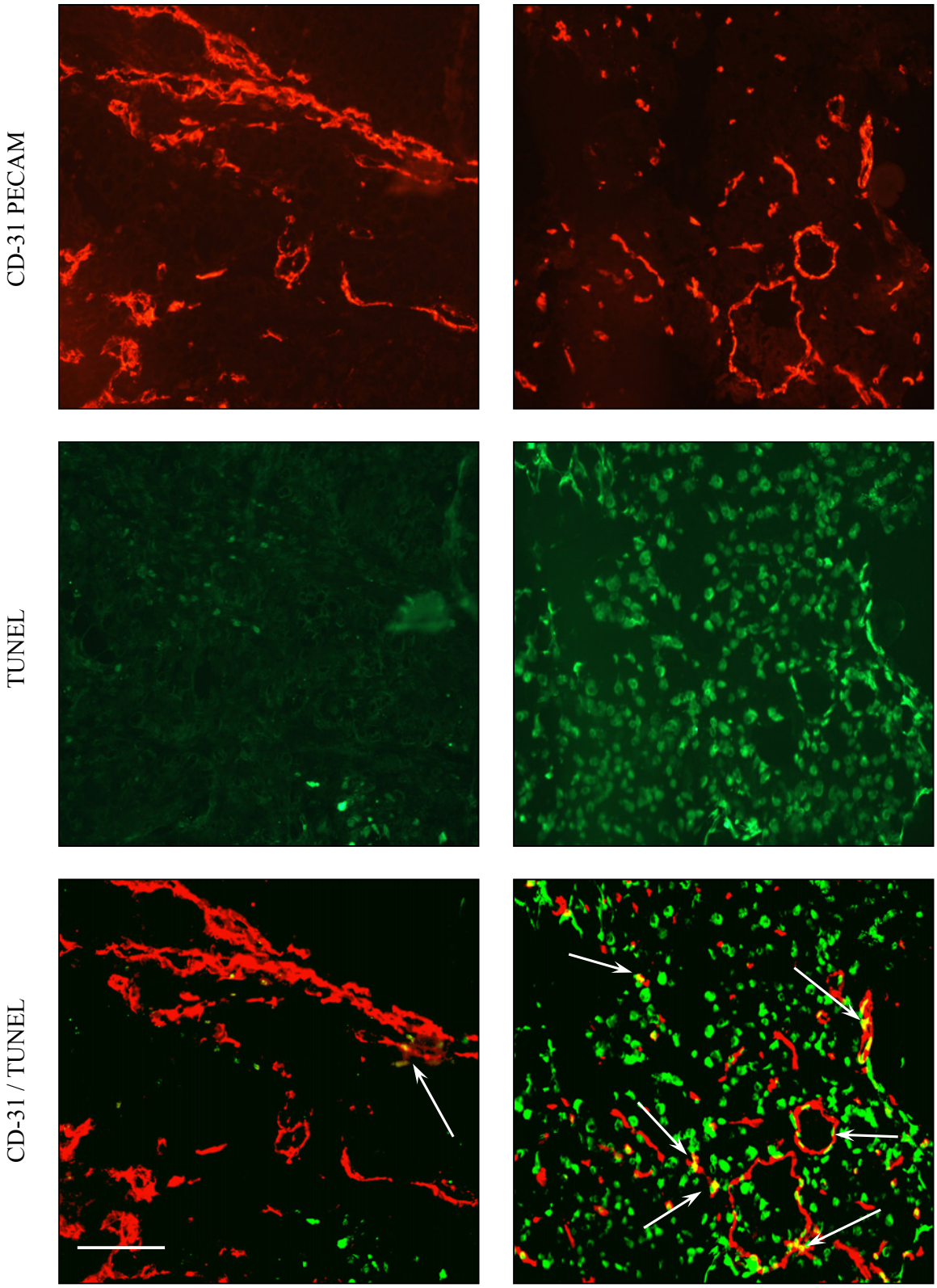

Control
AZM475271
CD-31 PECAM
TUNEL
CD-31 / TUNEL

Figure 6.6 Immunofluorescent staining of L3.6pl human pancreatic tumors from mice treated with AZM475271.

Serial tissue sections were stained with the immunofluorescent endothelial cell marker (CD31) and the immunofluorescent apoptosis marker (TUNEL). A representative sample (×100 magnification) of this CD31/TUNEL fluorescent double-staining is shown. *Fluorescent red*, CD31-positive endothelial cells; *fluorescent green*, TUNEL-positive cells; *fluorescent-yellow*, TUNEL-positive endothelial cells; bar = 100 µm, the arrows delineate TUNEL-positive endothelial cells.

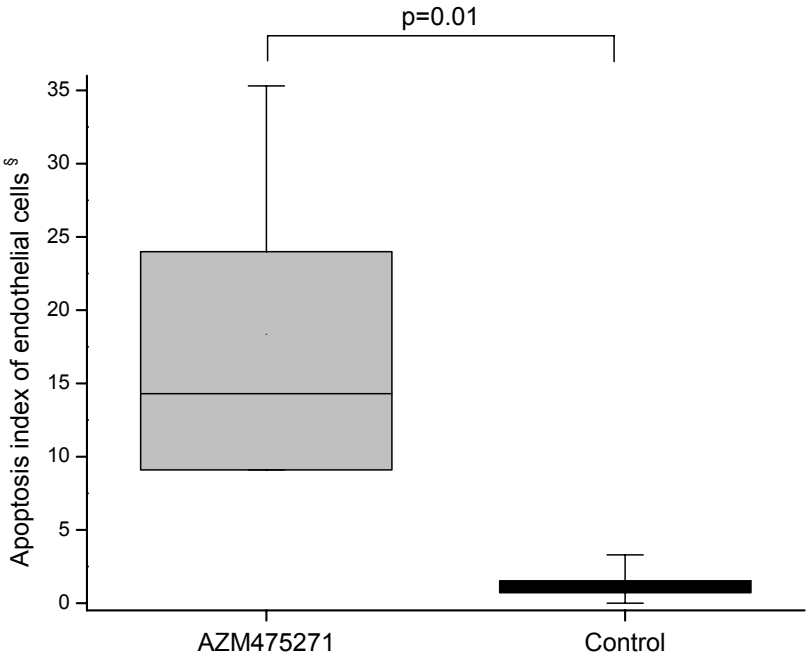


Figure 6.7 Apoptosis of endothelial cells of microvessels after therapy with AZM475271.

§ The total number of apoptotic cells was quantified and expressed as the ratio of apoptotic endothelial cells to the total number of endothelial cells in 5–10 random 0.011-mm^2 (at x400 magnification).

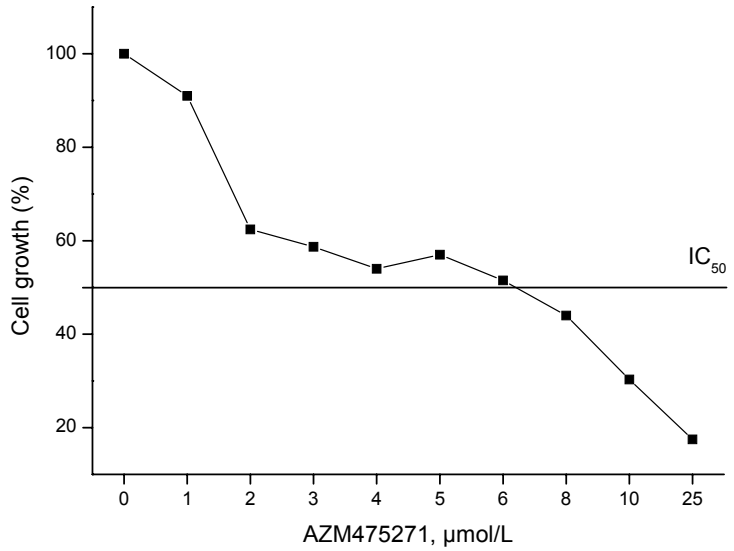
Box plots represent the means for each group with standard deviation of data (*min* and *max*) and standard error of the mean.

6.6 *In vitro* antiproliferative activity of AZM475271 in L3.6pl and HUVE cells

Viability assays for L3.6pl and HUVE cells were performed with the TACS MTT Cell Proliferation and Viability Assay Kit. Treatment of HUVECs with AZM475271 at high concentrations (> 10 µmol/L) caused a rapid decrease in cell proliferation that could be linked to the ability of AZM475271 to induce HUVEC death, whereas a significant antiproliferative activity of AZM475271 on HUVECs could be already observed at concentrations of 2 µmol/L or

above (IC_{50} = 6 µmol/L) (Fig. 6.8). Interestingly, no significant inhibition of proliferation was seen on L3.6pl cells at doses up to 5 µmol/L. The antiproliferative effect of AZM475271 on L3.6pl was seen exclusively at very high doses (IC_{50} = 25 µmol/L) (Fig. 6.8). These results suggest that the anti-angiogenic properties of AZM475271 might predominantly be mediated through a direct effect on the endothelial cells rather than *via* inhibition of tumor cells.

A



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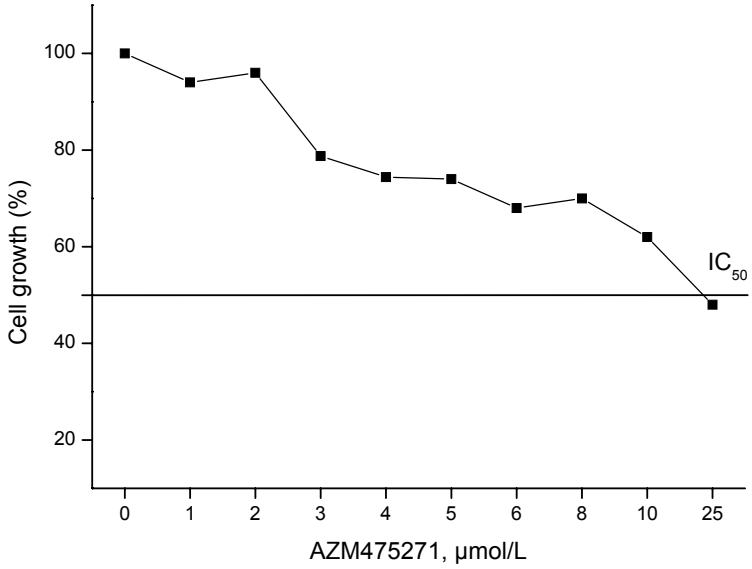


Figure 6.8 In vitro effects of AZM475271 on tumor and endothelial cell proliferation.

Viability assays for HUVE (a) and L3.6pl (b) cells with AZM475271 were performed with the TACS MTT Cell Proliferation and Viability Assay Kit. Treatment of HUVECs with AZM475271 at high concentrations (>10 μmol/L, $p<$ 0.0001 *versus* AZM475271 0 μmol/L) caused a rapid decrease in cell proliferation that could be linked to the ability of AZM475271 to induce HUVEC death. The IC_{50} concentration was reached at 6 μmol/L for HUVECs and at 25 μmol/L for L3.6pl cells, respectively.

6.7 *In vitro* effects of AZM475271 on VEGF- and EGF- dependent endothelial cell proliferation

The effects of exogenous VEGF or EGF on endothelial cell proliferation were analyzed using the TACS MTT Cell Proliferation and Viability Assay Kit.

To address the functional importance of HUVEC proliferation in response to growth factors, we incubated the cells for 48 hours in serum reduced medium (0.5% FCS) in the presence or absence of VEGF or EGF. Both growth factors at concentrations of 25 ng/ml induced endothelial cell proliferation under the starved conditions (Fig. 6.9). The results allow us to speculate that VEGF and EGF signalling pathways play a critical role in promoting endothelial cell survival under our *in vitro* conditions.

Cell proliferation assays were performed to examine an *in vitro* inhibition of VEGF and EGF induced HUVEC growth following treatment with AZM475271. Inhibition of Src kinase in HUVECs by AZM475271 showed prevention of survival signaling from VEGF – and EGF – induced cell growth in a dose-dependent cytostasis. Interestingly, the IC_{50} concentration for AZM475271 in both VEGF- and EGF- stimulated HUVECs was significantly higher (Fig. 6.10) than that found under unstimulated conditions (Fig. 6.8a). The results allow us to speculate that VEGF and EGF signalling pathways play a critical role in promoting endothelial cell proliferation.

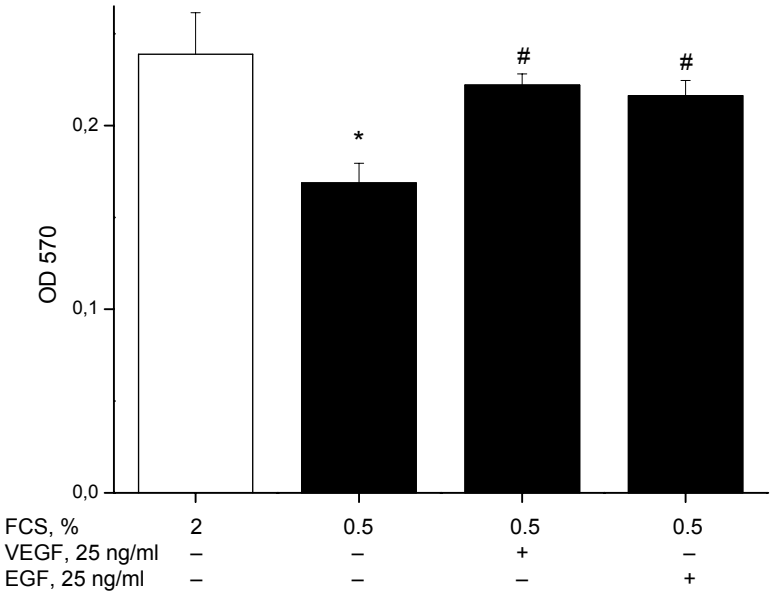
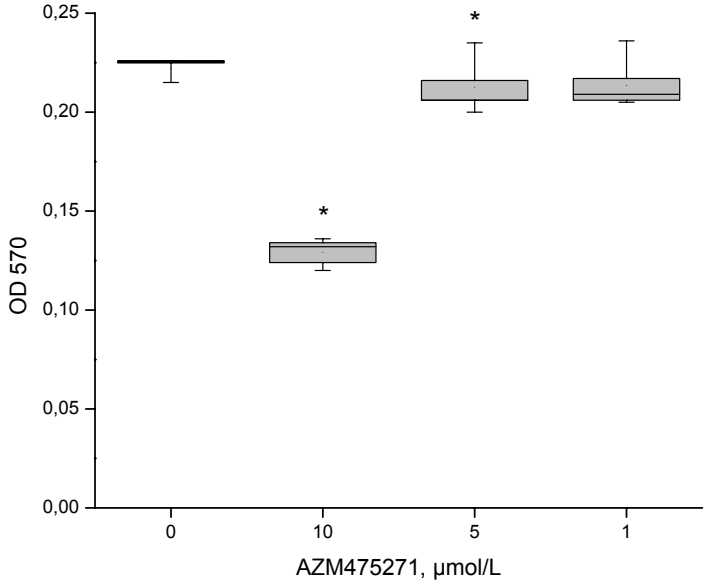


Figure 6.9 The effects of exogenous VEGF or EGF on endothelial cell proliferation.

Both growth factors at concentrations of 25 ng/ml induced endothelial cell proliferation under the starved conditions. (* $p<0.05$ compared with 2% FCS control, $^{\#}$ $p<0.05$ compared with 0.5% FCS starved control).

A



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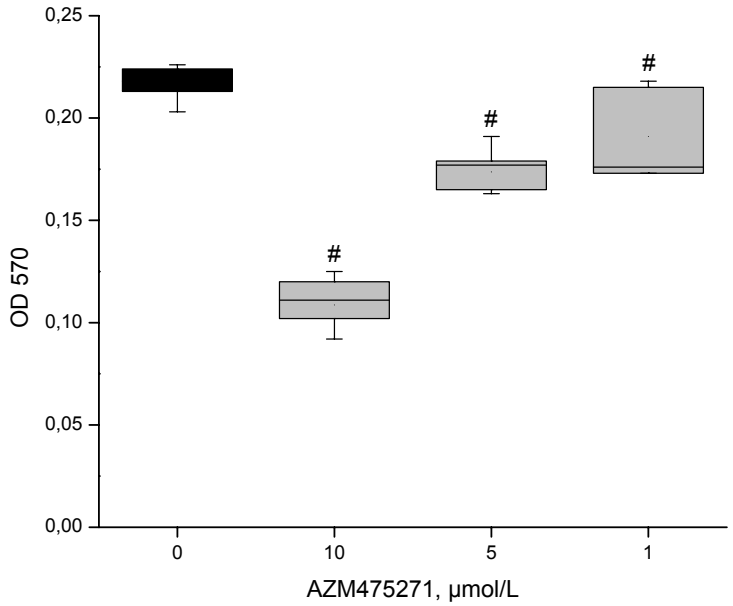
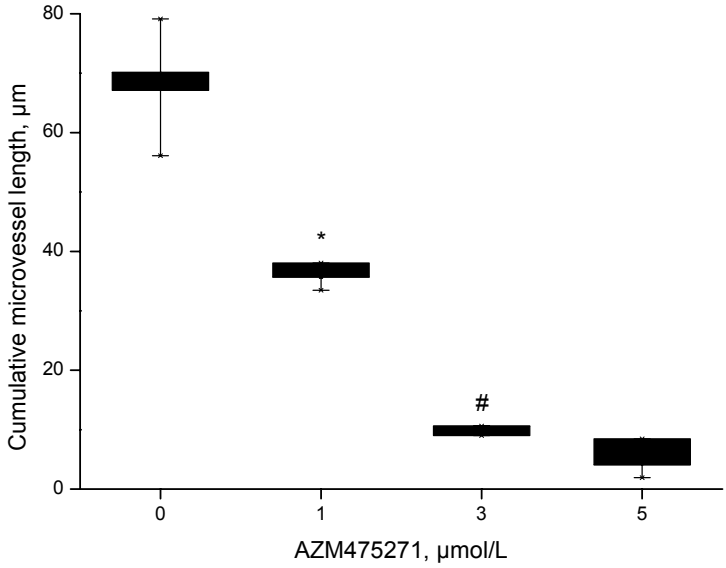


Figure 6.10 Inhibition of VEGF (A) – and EGF (B) – mediated endothelial cell proliferation.
Cells were treated with AZM475271 (1 – 10 µmol/L) in the presence of human recombinant $VEGF_{165}$ or human recombinant EGF at concentrations of 25 ng/ml in serum reduced Endothelial Cell Basal Medium. Inhibition of Src kinase in HUVECs by AZM475271 showed prevention of survival signaling from VEGF (A) – and EGF (B) – induced cell growth in a dose-dependent cytostasis. Box plots represent the means for each group with standard deviation of data (*min* and *max*) and standard error of the mean; *, $p< 0.05$ *versus* VEGF – stimulated cells; $^{\#}$, $p< 0.05$ *versus* EGF – stimulated cells.

6.8 *In vitro* effect of AZM475271 in the aortic ring assay

Angiogenesis was studied by culturing aortic explants in three-dimensional matrix gels according to the procedure of Nikosia and Ottineri. Endothelial cell sprouting from cultured rat aortic rings was abrogated by AZM475271 at any of the concentrations tested (1 µmol/l to 5 µmol/L; Fig. 6.11). At a concentration of 1 µmol/L endothelial migration and sprouting was 52 % relative to untreated controls ($p = 0.0024$ *versus* control). In contrast, a concentration of 5 µmol/L inhibited sprouting by 93 % relative to controls ($p < 0.0005$ *versus* control; Fig. 6.7).

A


Cumulative microvessel length, µm
80
60
40
20
0
*
#
0
1
3
5
AZM475271, µmol/L

B

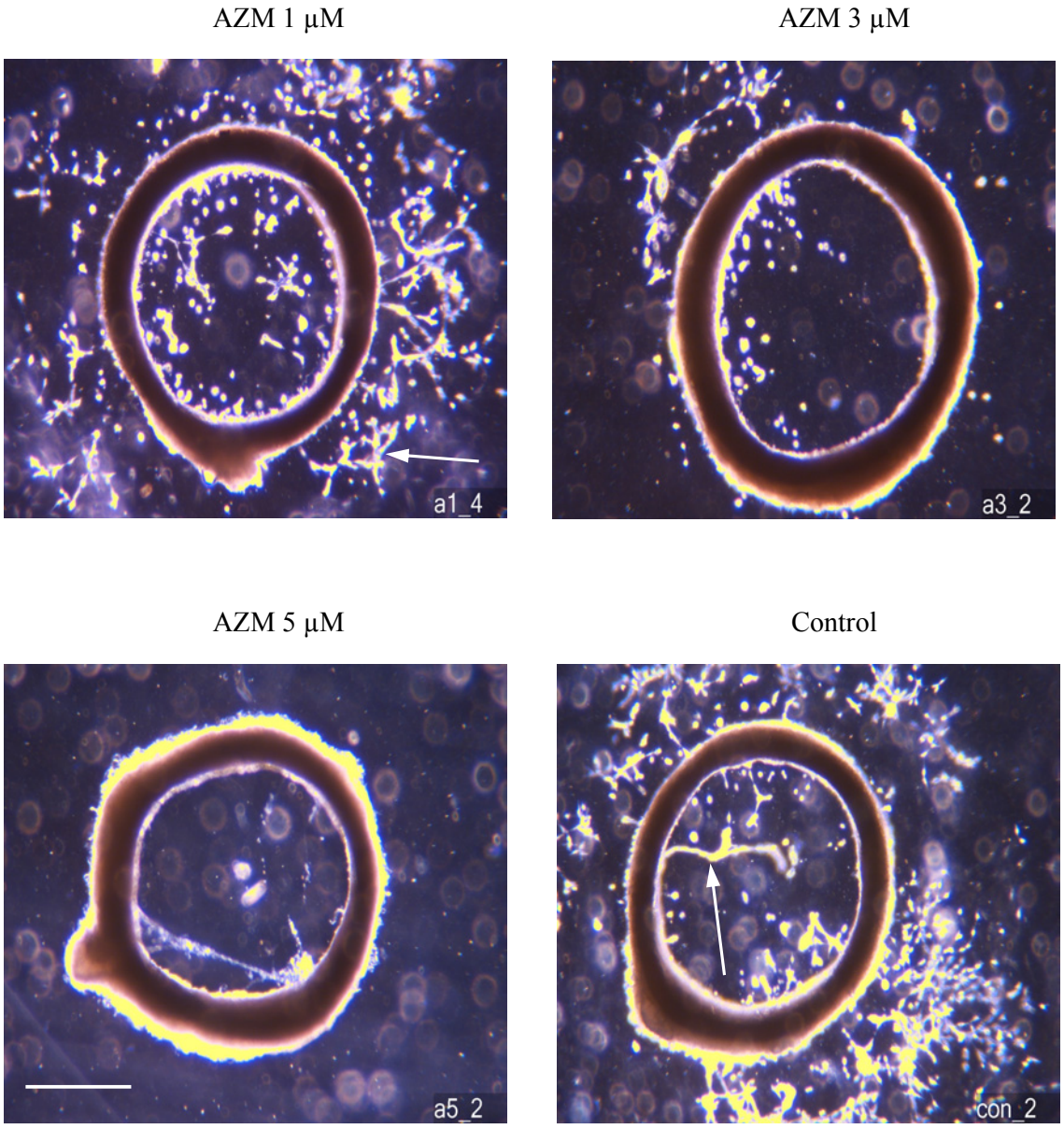


Figure 6.11 Effect of AZM475271 on rat aortic ring formation.
An estimation of the length of the capillary was performed by measuring the distance from the aortic explant to the approximate mean point of capillary. Endothelial cell sprouting from cultured rat aortic rings was abrogated by AZM475271 (a) at any of the concentrations tested (1 µmol/L to 5 µmol/L). At a concentration of 1 µmol/L endothelial migration and sprouting was 52 % relative to untreated controls. By contrast, a concentration of 5 µmol/L inhibited sprouting by 93 % relative to controls ($^{\#}$, $p<0.0004$ *versus* AZM475271 0 µmol/L; *, $p = 0.0024$ *versus* AZM475271 0 µmol/L). Representative photographs (b) showing the *in vitro* anti-angiogenic activity of the Src kinase inhibitor (the rings were examined by phase contrast microscopy with a Zeiss Axiotech Vario microscope at ×10 magnification, bar = 10 µm, the arrows delineate capillary outgrowth).

6.9 AZM475271 inhibits endothelial sprouting *in vitro* in the spheroid angiogenesis model

To investigate the complex processes of angiogenesis *in vitro*, we used the spheroid assay. We measured the ability of the endothelial cells to form sprouts in the spheroid model as a result of angiogenic stimulation, a prerequisite for an engagement of these cells in the formation of blood vessels.

Interestingly, AZM475271 inhibited sprouting of HUVEC at lower concentrations than suggested by the MTT proliferation assay (1 µmol/L for the sprouting assay *versus* 6 µmol/L for the MTT assay). At concentrations higher than the IC_{50}, sprouting of the endothelial cell spheroids was completely prevented (Fig. 6.12).

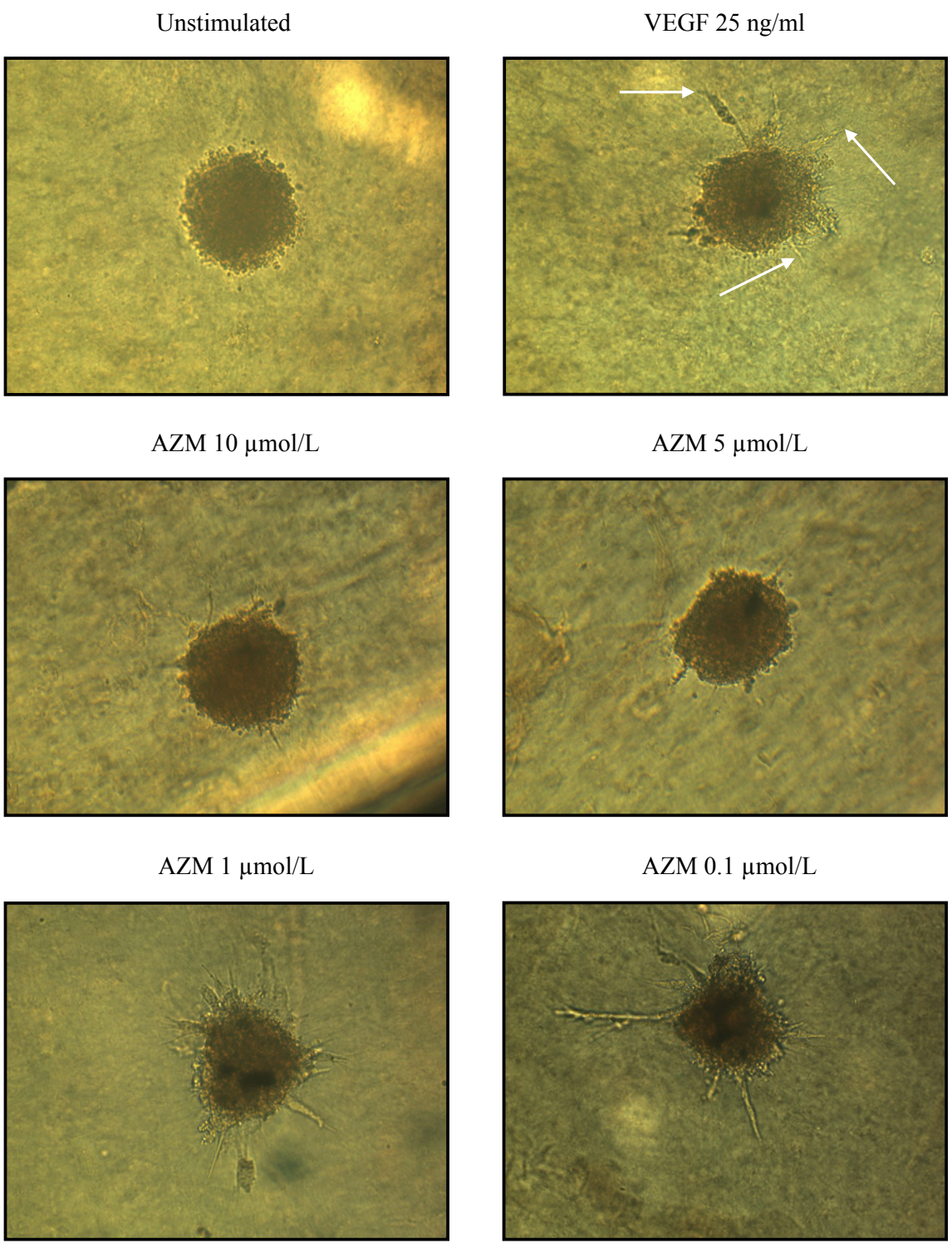


Figure 6.12 AZM475271 inhibits endothelial sprouting *in vitro* in the spheroid angiogenesis model

Spheroids were stimulated with 25 ng/ml VEGF-A_{165} and the inhibiting properties of AZM475271 were investigated. AZM475271 was able to overcome the stimulating effect of VEGF-A_{165} at micromolar concentrations. Representative

pictures of spheroids are presented (the pictures of the spheroids were taken under trans-illumination using an Achroplan objective providing a 20-fold magnification; the arrows delineate spheroid sprouting).

6.10 Inhibition of VEGF production by L3.6pl and HUVE cells following treatment with AZM475271

The level of VEGF was measured in cell culture supernates collected on day 0, 2, and 4 from L3.6pl or HUVE cells at one passage during treatment with Src kinase inhibitor AZM475271 at different concentrations (0.1 and 1 μmol/L). The assay was performed according to the manufacturer's recommendations. Absorbance at 450 nm was measured and corrected using the 540-nm reading on the microplate reader. VEGF content was calculated according to the parameter of the calibration curve. Calibration curves with a correlation coefficient at least 0.998 were used and data are expressed as picograms per millilitre per 10^6 cells. All experiments were replicated three times.

The concentrations of AZM475271 below or equal 1 μmol/L (the concentrations with a maximum of inhibitory effect against Src tyrosine kinase [Fig. 6.1] without antiproliferative activity on tumor or endothelial cells [Fig. 6.8]) were taken to show that the production of VEGF by tumor or endothelial cells is "Src-dependent". We found that the level of VEGF in HUVE cell supernate on Day 0 (the beginning of experiment) as well as the level of VEGF in Endothelial Cell Basal Medium completed with supplemented growth factors was mostly non-detectable. The level of VEGF in HUVE cell supernate increased on Day 4, although this difference did not reach a significant level (Fig. 6.13a).

The level of VEGF in supernate taken from L3.6pl cells was significantly higher (> 50-fold) compare to those found in the HUVE cell supernate on Day 2. The amount of VEGF expressing by L3.6pl cells on Day 4 after the beginning of experiment increased significantly (Fig. 6.13b).

Most interestingly, the blockade of Src kinase by AZM475271 dramatically reduced the level of VEGF in L3.6pl supernate, the effect which was found also in the cell culture supernate from HUVE cells (Fig. 6.13, a and b). The inhibition of VEGF production by L3.6pl and HUVE cells following treatment with AZM475271 was day- and dose- dependent.

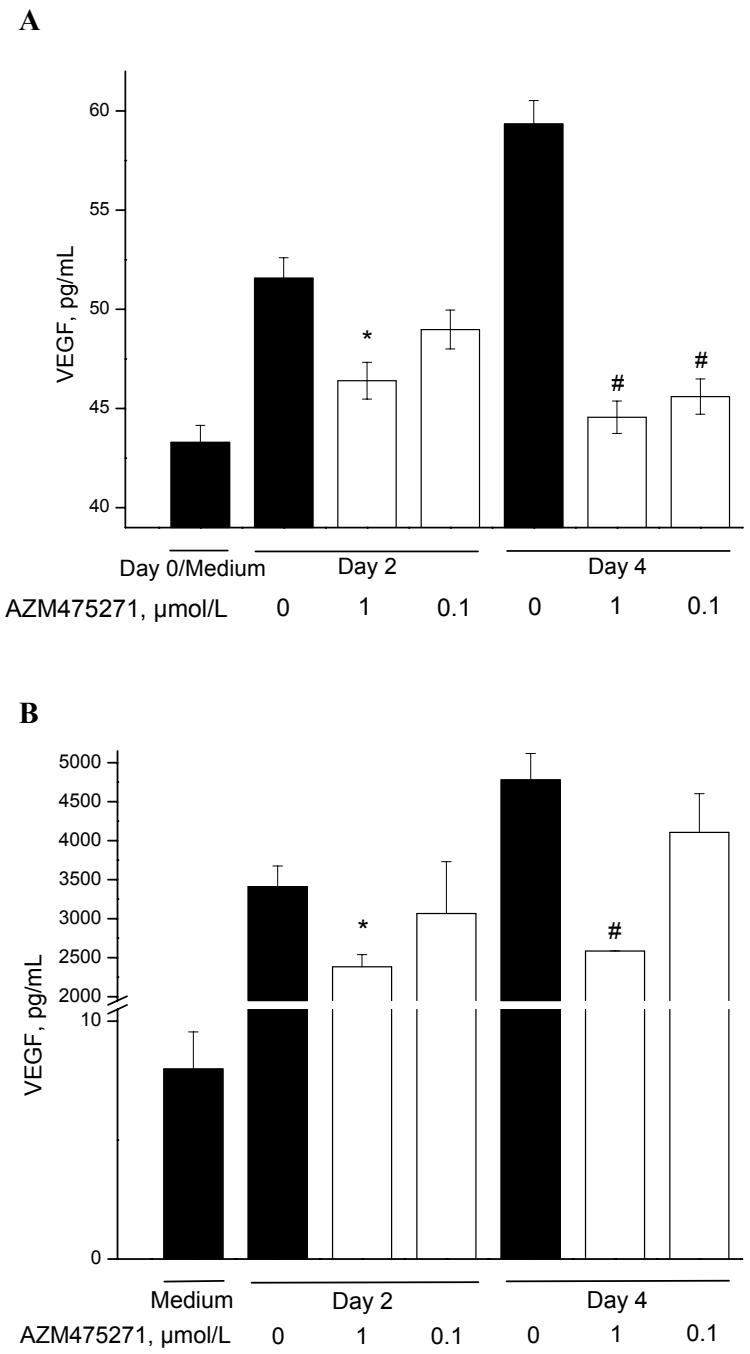
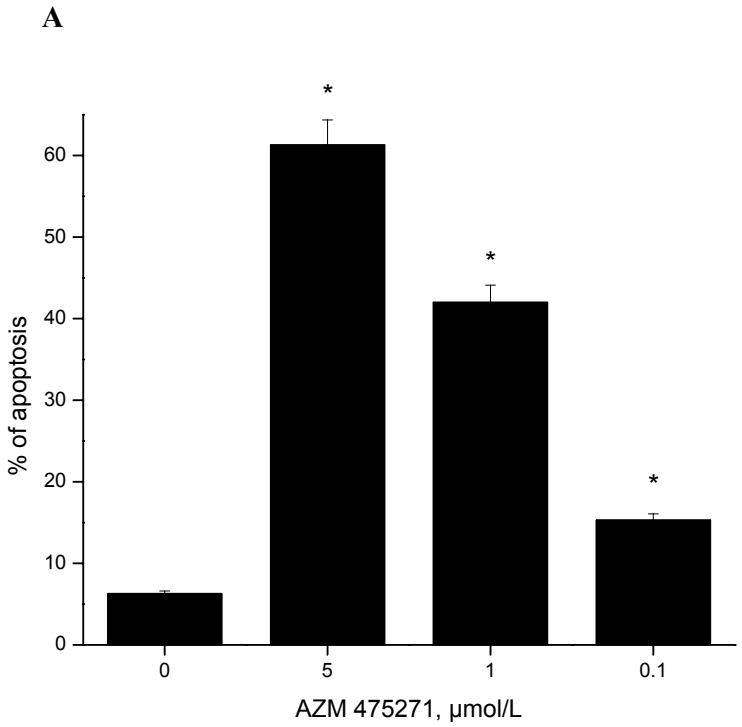


Figure 6.13 VEGF production by HUVE (a) and L3.6pl (b) cells following treatment with AZM475271.

The level of VEGF was measured in cell culture supernates collected on day 0, 2, and 4 from L3.6pl or HUVECs at one passage. The blockade of Src kinase by AZM475271 significantly reduced the level of VEGF in L3.6pl supernate in a dose-dependent manner (b), the effect which was found also in the cell culture supernate from HUVECs (a). (*, $p<0.05$ versus AZM475271 0 µmol/L, Day 2; $^{\#}$, $p<0.05$ versus AZM475271 0 µmol/L, Day 4).

6.11 *In vitro* induction of cell death in HUVECs

FACS analysis was used to determine the effects of AZM475271 on HUVEC cell cycle progression. Quantification of apoptosis was performed using propidium iodide staining. HUVECs were treated with AZM475271 in different concentrations for 12 hours, and then cells were collected and suspended in a Nicoletti buffer. Inhibition of Src kinase in HUVECs resulted in the induction of apoptosis in 99% of the cells treated with AZM475271 at concentrations from 10 up to 25 μM as compared with 6.3% of apoptotic cells present in the untreated control (Fig. 6.14, b). Interestingly, at the more physiologically relevant submicromolar doses of AZM475271 (5, 1 and 0.1 μmol/L) the level of apoptotic fraction was also significantly higher (60%, 40% and 15%, respectively) when compared with untreated cells (6%; Fig. 6.14, a).



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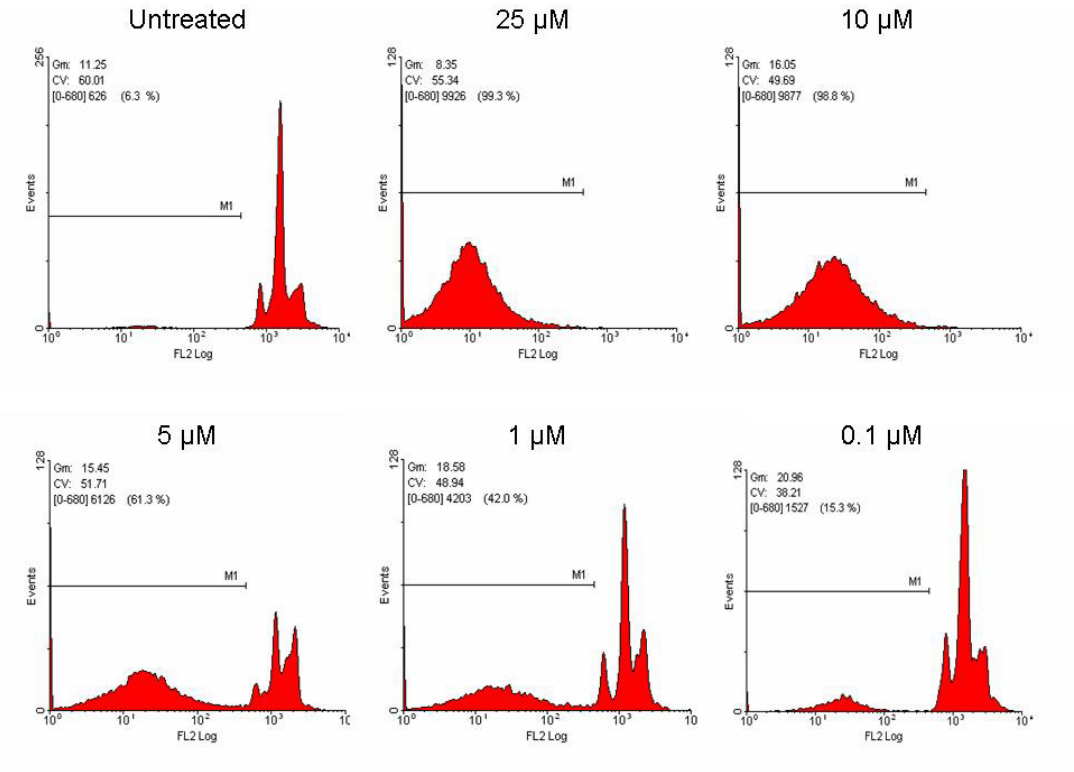

Figure 6.14 In vitro effect of AZM475271 on endothelial cell survival. Quantification of apoptosis was performed using propidium iodide staining for cell cycle analysis by FACS. AZM475271 promotes HUVEC apoptosis in a dose-dependent manner (a), (*, $p<0.05$ *versus* AZM475271 0 µmol/L). Histograms (b) show one representative of three independent experiments.

6.12 *In vitro* inhibition of migration of HUVECs by AZM475271

To investigate the anti-migratory effects of AZM475271 on endothelial cells we demonstrated whether Src inhibition in HUVECs prevented cell migration in a modified Boyden chamber assay. Inhibition of Src kinase resulted in a dose depended decrease of HUVEC migration. ($p < 0.05$; Fig. 6.15).

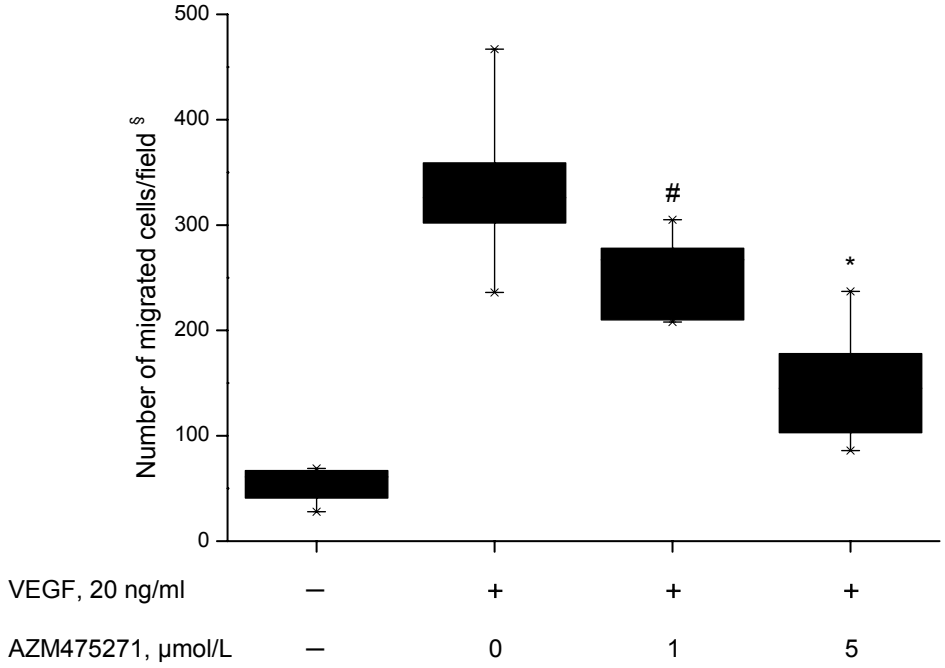

Figure 6.15 In vitro inhibition of migration of HUVECs by AZM475271. Migration of HUVECs was demonstrated using a modified Boyden chamber assay. Inhibition of VEGF-induced chemotaxis was assessed after including AZM475271 at relevant doses. Migration through the membrane was determined after 4 hours of incubation at 37^{o}C by fixing, staining with hematoxylin and eosin, and counting the migrated cells in five random fields at ×100 magnification. The average of triplicate inserts from three representative experiments was obtained. *Bars*, SD; *, p< 0.001; $^{\#}$, p< 0.0004 (both *versus* VEGF stimulation); §, positive cells in 5 random 0.159-mm^2 fields at x100 magnification.

6.13 AZM475271 abolishes VEGF-induced FAK phosphorylation in HUVECs

Src tyrosine kinase has been shown to mediate the tyrosine phosphorylation signal from VEGFR to FAK. Focal adhesion kinase is involved in integrin-mediated signal transduction. It plays an important role in the control of several biological processes, including cell spreading, migration and survival (Parsons et al., 2000). The recruitment of Src family kinases results in the phosphorylation of tyrosine residues 576 and 577 in the catalytic domain. Phosphorylation of tyrosines 576 and 577 is significantly elevated in the presence of c-Src *in vitro* and v-Src *in vivo* (Calalb et al., 1995). Furthermore, the maximal kinase activity of FAK immune complexes requires phosphorylation of both tyrosines 576 and 577.

Here, we test the possibility that AZM475271 could affect VEGF-induced FAK kinase activity in HUVECs. Using Western Blots, we found src-dependent up-regulation of FAK phosphorylation (Fig. 6.16). Treatment of HUVECs with 10 μmol/L of AZM475271 resulted in the significant inhibition of VEGF-activated pTyr 576/577 FAK phosphorylation, indicating that VEGF mediates its effect in HUVECs at least in part *via* Src kinase.

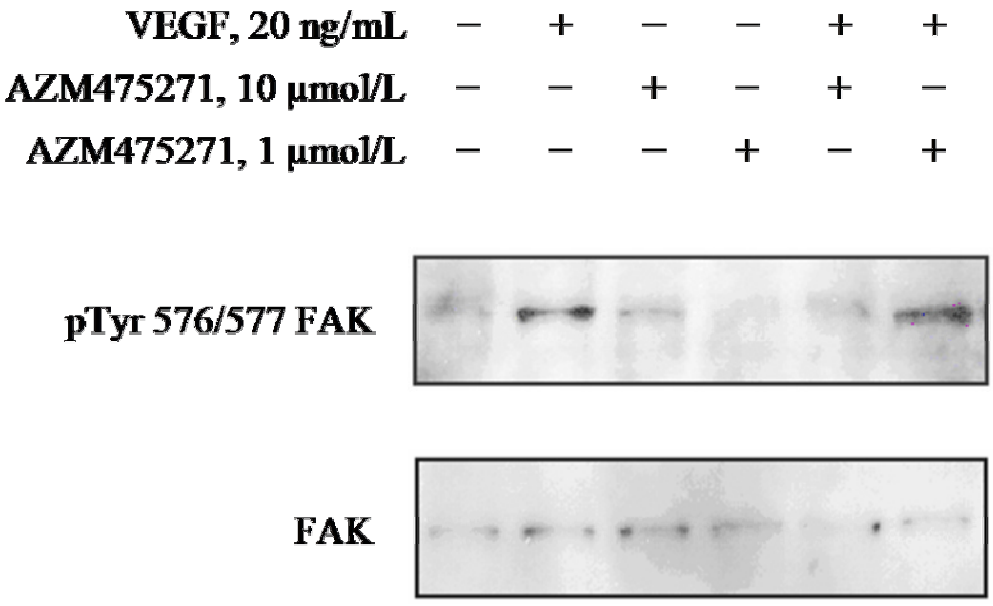

Figure 6.16 VEGF-induced HUVEC FAK phosphorylation is inhibited by AZM475271.
Lysates of VEGF-stimulated HUVECs were prepared as described in Materials and Methods and subjected to immunoblotting with anti-phosphotyrosine antibody specific for 576/577 within FAK.

Chapter 7

DISCUSSION

Currently, a large variety of chemotherapeutic drugs is being used to treat pancreatic cancer, but, unfortunately, many compounds show only limited efficacy due to problems of delivery and development of drug resistance in tumor cells. Tumor cells are rapidly changing targets because of their genetic instability, heterogeneity, and high rate of mutation, leading to selection and outgrowth of drug-resistant tumor cell populations (Gasparini et al., 1999; Kerbel, 1997).

Anti-angiogenic therapy offers several advantages over therapy directed against tumor cells. First, endothelial cells are a genetically more stable, diploid, and spontaneous mutations rarely occur. Second, the turnover of tumor endothelial cells is approximately 50 times higher than of endothelial cells in normal quiescent tissues. Third, tumor endothelial cells as target are easily accessible by systemic administration. Finally, a high number of tumor cells is sustained by a single capillary, that means, inhibition of a small number of tumor vessels may affect the growth of many tumor cells (Kerbel, 1997). It has been shown, that the intratumoral blood vessel density is a prognostic marker in a variety of solid tumors, including invasive breast (Kumar et al., 1999), lung (Fontanini et al., 1996), malignant melanoma, gastrointestinal, (Tanigawa et al., 1997) and genitourinary cancers (Pepper, 1997). The progressive growth of pancreatic neoplasms also depends on the induction of angiogenesis, in these tumors a positive correlation was found between tumor angiogenesis and the risk of metastasis, tumor recurrence, and death (Kumar et al., 1999; Fontanini et al., 1996; Tanigawa et al., 1997). However, as tumors grow, they begin to produce a wider array of angiogenic molecules. Therefore, if only one molecule (for example, VEGF) is blocked, tumors may switch to another molecule (for example, bFGF or IL-8). Thus we may require a cocktail of antibodies/inhibitors. In this respect, targeting intracellular proteins might be a promising approach in angiogenesis therapy of pancreatic cancer. Indeed, our results support the theory that the inhibition of intracellular transducers of receptor tyrosine kinase signaling can avoid these disadvantages.

The purpose of the current study was to determine whether the intracellular tyrosine kinase Src might play a role in angiogenesis of pancreatic cancer. c-Src is a multifunctional protein involved in the regulation of a variety of normal processes, including proliferation, differentiation, survival, motility, and functions of fully differentiated cells (Thomas and Brugge, 1997). There are several recent reports that Src contributes to the control of tumor angiogenesis. Specifically, Src is needed for hypoxia-induced vascular endothelial growth factor (VEGF) production in a number of cell types (Mukhadopathyay et al., 1995). Furthermore, it has been shown, that suppression of Src in HT29 colon cancer cells by an antisense approach, led to a reduction of VEGF expression (Ellis et al., 1998). Thus, it appears that Src cannot only regulate the production of VEGF, but also control the consecutive endothelial cell response (see Chapter 3).

To investigate the hypothesis, that Src inhibitory drugs do not only affect tumor cell proliferation and survival, but also angiogenesis and migration, we conducted an *in vivo* experiment using a novel orally available Src kinase inhibitor AZM475271 (AstraZeneca) in nude mice bearing L3.6pl human pancreatic cancer implanted orthotopically in the pancreas. Treatment with AZM475271 did not significantly change animal weight (at the end of experiment the mean animal body weight after therapy with AZM475271 was 21.6 g, compared to the untreated group with 22.6 g). Monitoring of the tumor volume over the time of experiment revealed a decrease in the tumor growth progression in all animals treated with AZM475271. The animals were sacrificed 32 days after the initiation of treatment, when > 50% of the control animals had become moribund. At the time of sacrifice, all animals developed primary pancreatic tumors, however the mean tumor volume was significantly decreased (~40% inhibition of primary tumor growth) from 1125 ± 460 mm^3 (control animals) to 508 ± 205 mm^3 in animals treated with AZM475271. Furthemore, treatment with AZM475271 significantly reduced spontaneous liver metastasis (no animals had metastases) compared with treatment using vehicle solution. Mice who received AZM475271 had also a significant reduction in the incidence of lymph node metastases (five of nine animals). In addition, daily intragastral administration of AZM475271 was well tolerated. IHC analyses of primary pancreatic tumors demonstrated a significant decrease in proliferating tumor cells, the mean number of *Ki-67* positive tumor cells after therapy with AZM475271 was reduced by > 50 % compared to control mice. The mean number of apoptotic cells in the tumors treated with AZM475271 was significantly elevated compared to control tumors (26 ± 11 *versus* 8 ± 2, respectively). The number of CD31-positive microvessels counted per x100 field in the pancreatic tumors was lower in mice after therapy with AZM475271, but not significant ($p = 0.14967$). We speculated that tumor cell apoptosis may

have been due to poor perfusion after endothelial cell apoptosis rather then due to decreasing in microvessel density. Indeed, double staining of endothelial cells with antibodies against CD31 and TUNEL revealed that the reduction in MVD was attributable to a significant increase of apoptosis in endothelial cells (24 ± 9.1 for AZM475271 compared with 0.7 ± 1.5 for untreated control, $p = 0.01$) and not to a direct effect on the tumor cells themselves (clusters of apoptotic tumor cells were seen surrounding apoptotic endothelial cells when treated with AZM475271). This finding suggests that the reduced tumor size that followed therapy with Src inhibitor was not wholly a direct consequence of inhibition of tumor cell proliferation. There was a statistically significant induction of endothelial cell apoptosis in the pancreatic tumor model when AZM475271-treated tumors and control tumors were compared. This is the first report to our knowledge demonstrating that therapy with AZM475271 leads to apoptosis of endothelial cells within pancreatic tumors as determined by a double-labeling immunofluorescence procedure. Moreover, the antiangiogenic activity of the Src kinase inhibitor was also evident *in vitro*, where proliferation, invasion, and migration of the endothelial cells were significantly reduced by AZM475271.

In vitro, AZM475271 was shown to inhibit proliferation and to induce apoptosis of human umbilical vein endothelial cells (HUVECs) in a dose dependent manner. Treatment of HUVECs with AZM475271 at high concentrations (> 10 µmol/L) caused a rapid decrease in cell proliferation that could be linked to the ability of AZM475271 to induce HUVEC death, whereas a significant antiproliferative activity of AZM475271 on HUVECs could be already observed at concentrations of 2 µmol/L or above (IC_{50} = 6 µmol/L). Interestingly, no significant inhibition of proliferation was seen on L3.6pl cells at doses up to 5 µmol/L. The antiproliferative effect of AZM475271 on L3.6pl was seen exclusively at very high doses (IC_{50} = 25 µmol/L). These *in vitro* findings support our theory that the anti-tumor properties of AZM475271 might predominantly be mediated through a direct effect on the endothelial cells rather that on the tumor cells.

Inhibition of Src kinase in HUVECs also showed prevention of survival signaling from VEGF and EGF. The IC_{50} concentration for AZM475271 in both VEGF- and EGF- stimulated HUVECs was higher (IC_{50} = 10 µmol/L) than that found in HUVECs under unstimulated conditions. The results allow us to speculate that VEGF and EGF signalling pathways play a critical role in promoting endothelial cell proliferation and survival. Indeed, it has been shown that receptor tyrosine kinases activate Src by autophosphorylation of tyrosine residues that function as docking sites for the SH2 domain of Src kinase on the receptor itself or by phosphorylation of docking proteins. Once activated, Src could link VEGF- or EGF-stimulation

with the PDK/PKB signaling cassette leading to stimulation of endothelial cell survival and angiogenesis (Schlessinger et al., 2000). However, although both growth factors stimulate Src activation, it has only been shown that VEGF-induced angiogenesis can be inhibited by treatment with a retrovirus that encodes for Src-251, a dominant-negative mutant of Src. Moreover, overexpression of Src-251 in avian blood vessels induces apoptotic death, indicating that VEGF-induced activation of Src is essential for endothelial cell survival and angiogenesis. Interestingly, VEGF-induced vascular permeability is impaired in $Src^{-/-}$ and in $Yes^{-/-}$ but not in $Fyn^{-/-}$ mice (Eliceiri et al., 1999), suggesting that the function of Src kinases in endothelial cells is not comparable to the function of other family members.

Furthemore, we found that in addition to regulating the VEGF response of HUVE cells, Src also regulates the expression of VEGF in both tumor and endothelial cells. The level of VEGF was measured in cell culture supernates collected on day 0, 2, and 4 from L3.6pl or HUVE cells at one passage during treatment with Src kinase inhibitor AZM475271 at different concentrations (0.1 and 1 μmol/L). The concentrations of AZM475271 below or equal 1 μmol/L (the concentrations with a maximum of inhibitory effect against Src tyrosine kinase without antiproliferative activity on tumor or endothelial cells) were taken to show that the production of VEGF by tumor or endothelial cells is "Src-dependent". We found that the level of VEGF in HUVE cell supernate on Day 0 (the beginning of experiment) as well as the level of VEGF in Endothelial Cell Basal Medium completed with supplemented growth factors was mostly non-detectable. The level of VEGF in HUVE cell supernate increased on Day 4, although this difference did not reach a significant level. The level of VEGF in supernate taken from L3.6pl cells was significantly higher (> 50-fold) compare to those found in the HUVE cell supernate on Day 2. The amount of VEGF expressing by L3.6pl cells on Day 4 after the beginning of experiment increased significantly. Most interestingly, the blockade of Src kinase by AZM475271 dramatically reduced the level of VEGF in L3.6pl supernate, the effect which was found also in the cell culture supernate from HUVE cells.

To gain more relevant information in terms of angiogenic processes, we measured the ability of the endothelial cells to form sprouts in the spheroid assay as a result of angiogenic stimulation, a prerequisite for an engagement of these cells in the formation of blood vessels. Cells organized in a spheroid that is embedded in a collagen matrix, are much more sensitive to pro-angiogenic as well as anti-angiogenic stimuli as compared to the same cells in a monolayer culture. This finding was also apparent in our experiments and is rationalized by the fact that proliferation is only one of the features of angiogenic processes. Additional processes such as cell plasticity and mobility are also pivotal features of angiogenesis. Therefore, our finding that AZM475271

abrogated the stimulating effect of VEGF-A_{165} even at submicromolar concentrations demonstrates the high susceptibility of sprouting endothelial cells to AZM475271.

Image analysis was also used to quantify angiogenesis on the rat aortic ring model by culturing aortic explants in three-dimensional matrix gels according to the procedure of Nikosia and Ottineri. This model bridges the gap between *in vivo* and *in vitro* models and is suitable to quantify spontaneous angiogenesis as well as to analyze a complex microvascular network induced by VEGF. Interestingly, endothelial cell sprouting from cultured rat aortic rings was abrogated by AZM475271 at any of the concentrations tested (1 µmol/l to 5 µmol/L). At a concentration of 1 µmol/L endothelial migration and sprouting was 52 % relative to untreated controls. In contrast, a concentration of 5 µmol/L inhibited sprouting by 93 % relative to controls.

Recent reports have demonstrated a major role for Src activity in the control of cell adhesion and cytoskeletal changes, which in turn regulate cell invasion and migration (Frame, 2002). To produce new vessels, endothelial cells must migrate, degrade extracellular matrix, divide, form tubes, and survive (Fidler et al., 1994; Folkman, 1995). Evidence is provided that VEGF activates Src kinase, which induces the phosphorylation of tyrosines 576/577 within FAK, facilitating the association of FAK with integrin $\alpha_v\beta_3$. Blockade of Src activity by AZM475271 inhibits the formation of a VEGF-induced FAK/ $\alpha_v\beta_3$ complex. Integrin $\alpha_v\beta_3$ is known to bind a number of ECM proteins, among them vitronectin, fibrinogen, von Willebrand factor, fibronectin and denatured collagen. More recent studies show that integrin $\alpha_v\beta_3$ can also bind directly to matrix metalloproteinase 2, thereby localizing the MMP-2-mediated matrix-degradation capacity to the invasive/migratory site of vascular cells during angiogenesis (Brooks et al., 1996). This enables endothelial cells to degrade and remodel the ECM during their invasion.

We confirmed the antimigratory effect of AZM475271 by analysis of endothelial cell migration using a modified Boyden chamber assay revealing a strong inhibition of HUVEC migration. These findings suggest that one major role of Src activity is to cooperate with activated growth factor receptors to induce adhesion and cytoskeletal changes that are associated with invasion and metastasis. Therefore, it is possible, that Src-specific inhibitory drugs, such as AZM475271, might primarily affect these pathways in endothelial cells, and so be useful in preventing further tumor spread, while not necessarily having such a strong influence on cancer cell growth.

Chapter 8

SUMMARY

The results of all experiments can be summarized as follows:

- AZM475271 is the selective inhibitor of the Src tyrosine kinase activity in HUVE and L3.6pl cells with the IC_{50} = 0.1 µmol/L;
- Monitoring of the tumor volume in mice bearing human pancreatic cancer revealed a decrease in the tumor growth progression in all animals treated with AZM475271;
- Daily intragastral administration of AZM475271 was well tolerated;
- At the time of necropsy, the mean tumor volume was significantly decreased (~40% inhibition of primary tumor growth) in all mice treated with AZM475271;
- Treatment with AZM475271 significantly reduced spontaneous liver metastasis (no animals had metastases) compared with treatment using vehicle solution. Mice who received AZM475271 had also a significant reduction in the incidence of lymph node metastases (five of nine animals);
- The mean number of proliferating tumor cells (*Ki-67*-positive) in harvested tumors was reduced over than 50 % in the AZM475271 therapy group compared to the control group;
- The mean number of apoptotic cells (measured by TUNEL staining) was significantly higher in the AZM475271 therapy group;
- The number of endothelial cells undergoing apoptosis (determined by a double CD31 and TUNEL staining) was significantly greater in pancreatic tumors from mice treated with AZM475271 compared to control tumors;
- A significant antiproliferative activity of AZM475271 on HUVECs can be already observed at concentrations of 2 µmol/L or above (IC_{50} = 6 µmol/L);
- Treatment of HUVECs with AZM475271 at high concentrations (> 10 µmol/L) caused a rapid decrease in cell proliferation that could be linked to the ability of AZM475271 to induce HUVEC death (measured by MTT proliferation assay);

- Inhibition of Src kinase in HUVECs by AZM475271 showed prevention of survival signaling from VEGF – and EGF – induced cell growth in a dose-dependent manner (measured by MTT proliferation assay);
- Endothelial cell sprouting from cultured rat aortic rings was abrogated by AZM475271 at any of the concentrations tested (1 μmol/l to 5 μmol/L);
- AZM475271 inhibited sprouting of HUVEC in the spheroid angiogenesis model at lower concentrations than suggested by the MTT proliferation assay (1 μmol/L for the spheroid assay *versus* 6 μmol/L for the MTT assay).
- The blockade of Src kinase by AZM475271 dramatically reduced the level of VEGF in L3.6pl and HUVEC supernates;
- Src inhibition in HUVECs prevented VEGF – induced cell migration in the modified Boyden chamber assay;
- Treatment of HUVECs with AZM475271 resulted in the significant inhibition of VEGF-activated pTyr 576/577 FAK phosphorylation.

Taken together, our results suggest that the Src kinase inhibitor AZM475271, in addition to its antiproliferative effects on pancreatic tumor cells, suppresses tumor growth *in vivo* potentially by anti-angiogenic mechanisms by inducing intratumoral endothelial cell apoptosis and finally reducing the MVD. It is an exciting prospect that Src inhibitory drugs might not only affect more traditional Src activities, such as cell growth, migration, and invasion, but could also induce endothelial cell death in regions of pancreatic tumors where neovascularisation is occurring. These findings are consistent with our *in vitro* studies, where AZM475271 was shown to significantly inhibit VEGF-induced endothelial cell proliferation, migration, spreading, and survival. VEGF production by L3.6pl cells was also inhibited following treatment with AZM475271, so angiogenesis inhibition found *in vivo* may be mediated, at least in part, by paracrine effects.

Since AZM475271 is well tolerated when given to animals, we believe that the findings of the present study may shed light on the pharmacological basis for the clinical application of Src kinase inhibitors for suppression of angiogenesis, which plays a crucial role in pancreatic tumor growth and metastasis.

Chapter 9

ZUSAMMENFASSUNG

Die Ergebnisse der Doktorarbeit können wie folgt zusammengefasst werden:

- AZM475271 ist ein selektiver inhibitor der Scr Tyrosinkinase-Aktivität in humanen vasklären Endothelzellen undin humanen Pankreaskarzinomzellen L3.6pl mit einem IC_{50} = 0,1µmol/l
- Durch regelmäßiges Monitring konnte im Tierexperiment eine Abnahme des Pankreastumorwachstums nach Behandlung mit AZM475271 festgestellt werden
- Die tägliche intragastrale Verabreichung von AZM475271 wurde von allen Tieren gut vertragen
- Zum Zeitpunkt der Tötung war das Tumorvolumen nach Behnadlung mit AZM475271 signifikant niedriger (~ 40% niedriger)
- Die Behandlung mit AZM475271 führte zu einer signifikanten Reduktion der Inzidenz von Leber- und Lymphknotenmetastasen
- Die Anzahl der proliferierenden Zellen (*Ki*67 positiv) in den Pankreastumoren war nach Behandlung mit AZM475271 bis zu 50% im Vergelich zu den Kontrolltieren reduziert
- Die mittlere Anzahl apoptotischer Zellen (TUNEL-Färbung) in den Pankreastumoren war signifikant höher nach Therapie mit AZM475271 als in den Kontrolltumoren
- Die mitlere Anzahl apoptotischer Endothelzellen (TUNEL/CD31-Doppelfärbung) in den Pankreastumoren war signifikant höher nach Therapie mit AZM475271 als in Kontrolltumoren
- In HUVECs zeigt sich bereits ab einer Konzentration von 2µmol/l (IC_{50} = 6µmol/l) ein signifikant anti-prolferativer Effekt im MTT-Assay
- Die Behandlung von HUVECs mit hohen Dosen von AZM475271 (>10µmol/l) führt zum Zelltod
- Die Blockade der Src Kinase in HUVECs durch AZM475271 zeigte eine dosis-abhängige Inhibition des VEGF- und EGF induzierten Zellwachstums

- Das endotheliale „Sprouting“ kultivierter Rattenaortenringe konnte mit AZM475271 in Dosieurungen von 1-5µmol/l unterbunden werden.
- AZM475271 führte zur Hemmung des „Sproutings“ im Spheroid-Angiogenese-Assay
- Die Blockade der Src Kinase mit AZM475271 führte zur deutlichen Reduktion der VEGF-Konzentrationen in Überständen von kultivierten L3.6pl und HUVE-Zellen
- Src Inhibition in HUVECs verhindert VEGF-induzierte Zellmigration in eine modifizieren Boyden Chamber Assay
- Die Behandlung von HUVECs mit AZM475271 resultierte in einer signifikanten Inhibition der VEGF-aktivierten pTyr576/577 FAK-Phosphorylierung

Schlußfolgernd lassen diese Ergebnisse vermuten, daß die Reduktion des Pankreastumorwachstums durch den Src Kinase inhibitor AZM475271 zum einen durch den anti-proliferativen Effekt auf die Pankreastumorzellen bedingt ist, desweiteren allerdings durch einen potentiell anti-angiogenetischen Mechanismus durch Induktion der intratumoralen Apoptose von Endothelzellen und damit Reduktion der Mikrogefäßdichte im Tumor. Das Spektrum Src-inhibierender Substanzen beschränkt sich daher nicht nur auf ihre anti-migratorische, anti-invasive oder anti-proliferative Wirkunsgweisen, sondern kann auch auf anti-angiogenetische Effekte erweitert werden.

Die *in vivo* Ergebnisse im orthotopen Pankreastumor-Mausmodell sind in den *in vitro* Experimenten nachvollziehbar: der Src Kinase Inhibitor AZM475271 führte zur signifikanten Reduktion des VEGF-induzierten Überlebens, der Proliferation, Migration und des „Sproutings“. Die tumorzelleigene VEGF-Produktion konnte durch den Src Kinase Inhibitor AZM475271 deutlich reduziert werden, so daß anzunehmen ist, daß die Angiogenese-Inhibition auch zum Teil durch parakrine Effekte vermittelt wird.

Da AZM475271 im Tierexperiment gut verträglich war, ist vorstellbar, daß Src Kinase Inhibitoren nach Kenntnis ihrer pharmakologischen Effektivität – auch als anti-angiogenetische Substanz – eine klinische Applikation insbesondere beim metastasierten Pankreaskarzinom finden könnten.

Chapter 10

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Chapter 11

ABBREVIATIONS

5-FU	5-Fluorouracil
Ang1	Angiopoietin 1
APS	Ammonium-persulfate
ATP	Adenosine triphosphate
AUR-3	Aurora kinase
BCA	Bicinchoninic acid
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumine
CDK-2	Cyclin-dependent kinase-2
cDNA	Complementary deoxyribonucleic acid
Chk	Csk-homologous kinase
CSF1-R	Colony stimulation factor-1 receptor
Csk	C-terminal Src kinase
DAPI	4',6-diamino-2-phenylindole
dFdCDP	Gemcitabine-diphosphate
dFdCTP	Gemcitabine-triphosphate
DMEM	Dulbecco's modified Eagles medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dTMP	Deoxythymidine-5'-monophosphate
DTT	D,1-Dithiothreitol
dUMP	Deoxyuridine-5'-monophosphate
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediamine-tetra acetic acid
EGF	Epidermal growth factor
ERK	Extracellular regulated kinase
FACS	Fluorescence-activated cell sorting
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FCS	Fetal calf serum
FGF	Fibroblast growth factor

Flt-1	fms-like tyrosine kinase 1
Flt3	Fms like tyrosine kinase-3
GDP	Guanosine 5'-Diphosphate
GTP	Guanosine 5'-Triphosphate
H&E	Haematoxylin and eosin
HGF	Hepatocyte growth factor
HRP	Horseradish peroxidase
HUVEC	Human umbilical vein endothelial cells
Ig	Immunoglobulin
IGF	Insulin growth factor
IL	Interleukin
JAK	Janus kinase
JM	Juxtamembrane
KDR	Kinase insert domain-containing receptor
Lck	Lymphocyte-specific protein tyrosine kinase
LN	Lymph node
Lyn	Lck/Yes related novel tyrosine kinase
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MVD	Microvessel density
NF-κB	Nuclear factor-κB
PAGE	Polyacrylamid gel electrophoresis
PDGF	Plateled-derived growth factor
PI	Propidium iodide
PI3-K	Phosphatidylinositol 3-kinase
PKB	Protein kinase B
PKC	Protein kinase C
PML	Promyelocytic leukemia
PMSF	Phenylmethylsulfonyl fluoride
PP2	4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine
PTK	Protein tyrosine kinase
PTP	Protein tyrosine phosphatase
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase
SDS	Sodium dodecyl sulfate
SFK	Src family kinase
SH	Src homology
Src	Raus sarcoma virus proto-oncogene product
STAT	Signal transducers and activators of transcription
TEMED	N,N,N',N'-Tetra-methylethylenediamine

TGF-β	transforming growth factor-β
TKD	Tyrosine kinase domain
TMB	3,3′,5,5′-Tetramethylbenzidine
TNF-α	tumor necrosis factor-α
TS	Thymidylate synthase
TUNEL	Terminal deoxynucleotidyl transferase-mediated nick end labeling
VEGF	Vascular endothelial growth factor
Yes	Yamaguchi 73 and Esh avian sarcoma

Chapter 12

CURRICULUM VITAE

Personal data

Family name:	Ischenko
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	Germany
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Education

1986-1994	Primary school in Donetsk, Ukraine
1994-1996	Secondary school in Donetsk, Ukraine

Academic education and scientific experience

1996-2002	Studies of medicine at the Faculty of Medicine, Donetsk State Medical University, Ukraine
2002-2003	Clinical training in the Department of Vascular Surgery at the University of Hannover (MHH), Germany
2003-present	MD, graduate student Supervisor: PD Dr. Christiane J. Bruns

Professional skills

Basic cell culture techniques

In vitro and *in vivo* development of drug-resistant cell lines

Detection of viable cells

BrdU incorporation assay

Invasion and chemotaxis methods

Cell cycle analyses

Determination of apoptotic cells by FACS analysis

Enzyme-linked immunoassays

Immunoprecipitation analysis

Western blot analysis

In vitro kinase assays

Proteomics

Plasmid DNA isolation

Transfection techniques

Total RNA extraction and RT-PCR

Aortic ring assay

Spheroid angiogenesis assay

In vitro wound-healing assay

Murine Matrigel™ plug assay

In vivo allograft and xenograft tumor models

In vivo examination of antitumor activity

In vivo determination of tissue hypoxia

Immunohistochemistry of paraffin embedded tissues

Immunohistochemistry of snap-frozen tissues

Immunofluorescence staining and microscopy

Quantification of immunohistochemical and immunofluorescence tissue staining

In vivo gene delivery of nacked DNA *via* hydrodynamic injection technique

Participation in scientific meetings

Posters:

2003	15th EORTC-NCI-AACR Symposium on Molecular Targets and Cancer Therapeutics, November 18-21, 2003, Boston, USA Bruns, CJ., Yezhelyev M., **Ischenko I**., Guba M., Jauch K.-W., Ryan A., Barge A., Green T., Fennell M. Synergistic effect of the Src kinase inhibitor AZM475271 and gemcitabine in human pancreatic cancer growing orthotopically in nude mice
2004	8th Annual Meeting on Surgical Research (Chirurgische Forschungstage), 27-30 October, Mannheim, Germany **Ischenko, I**., Yezhelyev, M., Papyan, A., Guba, M., Jauch, KW., Bruns, CJ. Effect of Src Kinase Inhibition on Metastasis and Tumor Angiogenesis in Human Pancreatic Cancer
2004	8th Annual Meeting on Surgical Research (Chirurgische Forschungstage), 27-30 October, Mannheim, Germany Schmid, G., Guba, M., Papyan, A., **Ischenko, I**., Brückel, M., Bruns, CJ., Jauch, KW., Graeb, C. Administration of FTY720 Inhibits Angiogenesis and reduces Tumor Growth
2004	First European Conference on Tumor Angiogenesis and Antiangiogenic Therapy, 1-3 October, Nymphenburger Chateau Munich, Germany Papyan, A., Werner,A., **Ischenko, I**., Teifel, M., Michaelis, U., Jauch, KW, Bruns, CJ. Combination of Standard Chemotherapy with MBT-0206 Enhances the Anti-tumor Efficacy in a Highly Metastatic Human Pancreatic Cancer Mouse Model
2004	First European Conference on Tumor Angiogenesis and Antiangiogenic Therapy, 1-3 October, Nymphenburger Chateau Munich, Germany **Ischenko, I**., Yezhelyev, M., Papyan, A., Guba, M., Jauch, KW., Bruns, CJ. Effect of Src Kinase Inhibition on Metastasis and Tumor Angiogenesis in Human Pancreatic Cancer
2004	American Association for Cancer Research, 95th Annual Meeting, 27-31 March, Orlando, USA

Bruell, D., Yezhelyev, M., **Ischenko, I**., Huhn, M., Bruns, CJ., Brabletz, T., Fischer, R., Finnern, R., Barth, S. Specific Cytotoxic Activity of a Recombinant Anti-EGF Receptor Immunotoxin 425(scFv)-ETA towards a Highly Metastatic Pancreatic Carcinoma Cell Line

2004 American Association for Cancer Research, 95th Annual Meeting, 27-31 March, Orlando, USA

Yezhelyev, M., **Ischenko, I**., Guba, M., Ryan, A., Barge, A., Jauch, K-W., Green, T., Fennell, M., Bruns, CJ. Synergistic Effect of the Src Kinase Inhibitor AZM475271 and Gemcitabine in Human Pancreatic Cancer Growing Orthotopically in Nude Mice

2004 American Association for Cancer Research, 95th Annual Meeting, 27-31 March, Orlando, USA

Papyan, A., **Ischenko, I**., Werner, A., Yezhelyev, M., Teifel, M., Michaelis, U., Bruns, CJ. MBT-0206 Enhances the Anti-tumor Efficacy in a Highly Metastatic Human Pancreatic Cancer Mouse Model

2004 Kongress der Deutschen Gesellschaft für Chirurgie, 27-30 April, Berlin, Germany

Papyan, A., Werner, A., **Ischenko, I**., Teifel, M., Michaelis, U., Jauch, K-W., Bruns, CJ. Combination of Standard Chemotherapy with MBT-0206 Enhances the Anti-tumor Efficacy in a Highly Metastatic Human Pancreatic Cancer Mouse Model

2005 American Association for Cancer Research, 96th Annual Meeting, 16-20 April, Anaheim, USA

Bruns, CJ., **Ischenko, I**., Papyan, A., Guba, M., Green, T., Fennel, M., Jauch, K-W. Effect of Src Kinase Inhibition on Metastasis and Tumor Angiogenesis in Human Pancreatic Cancer

2005 9th Annual Meeting on Surgical Research (Chirurgische Forschungstage), 19-21 September, Frankfurt, Germany

Ischenko, I., Yezhelyev, M., Guba, M., Schmid, G., Jauch, K-W., Bruns, CJ. Inhibition of Src Tyrosine Kinase by AZM475271 Enhances the Efficacy of 5-Fluorouracil and Gemcitabine in Human Pancreatic Carcinoma Cells

2005 9th Annual Meeting on Surgical Research (Chirurgische Forschungstage), 19-21 September, Frankfurt, Germany

	Schwarz, B., **Ischenko, I**., Guba, M., Schmid, G., Jauch, K-W., Bruns, CJ. Blokade of Src Tyrosine Kinase Inhibits Angiogenesis and Reduces Growth of Pancreatic Carcinoma *in vivo*
2005	9th Annual Meeting on Surgical Research (Chirurgische Forschungstage), 19-21 September, Frankfurt, Germany
	Schmid, G., Guba, M., Papyan, A., **Ischenko, I**., Brückel, M., Bruns, CJ., Jauch, KW., Graeb, C. Administration of FTY720 Inhibits Angiogenesis and reduces Tumor Growth
2006	ASCO Gastrointestinal Cancers Symposium, 26-28 January, San Francisco, USA
	Ischenko, I., Čamaj, P., De Toni, E., Heeschen, C., Jauch, C-W., Bruns, CJ. The Effect of Src Kinase Inhibition on 5-Fluorouracil Chemosensitivity Is Related to Thymidylate Synthase Expression in Human Pancreatic Carcinoma Cells
2006	Pancreatic Cancer 2006, 1-3 June, Munich, Germany
	Ischenko, I., Čamaj, P., De Toni, E., Heeschen, C., Jauch, C-W., Bruns, CJ. The Effect of Src Kinase Inhibition on 5-Fluorouracil Chemosensitivity Is Related to Thymidylate Synthase Expression in Human Pancreatic Carcinoma Cells
2006	10th Annual Meeting on Surgical Research (Chirurgische Forschungstage), 21-23 September, Muenster, Germany
	Ischenko, I., Čamaj, P., De Toni, E., Heeschen, C., Jauch, C-W., Bruns, CJ. The Effect of Src Kinase Inhibition on 5-Fluorouracil Chemosensitivity Is Related to Thymidylate Synthase Expression in Human Pancreatic Carcinoma Cells
2006	10th Annual Meeting on Surgical Research (Chirurgische Forschungstage), 21-23 September, Muenster, Germany
	Conrad, C., Valesky, M., Huss, R., **Ischenko, I**., Mojaat, A., Jauch, K-W., Nelson, PJ., Bruns, CJ. Homing of circulating MSC (CD 34-) in regenerating liver and hepatic coloncarcinoma metastases
2006	Second European Conference on Tumor Angiogenesis and Antiangiogenic Therapy, September, Munich, Germany
	Ischenko, I., Schmid, G., Huber, S., Guba, M., Jauch, K-W., Bruns, CJ. Involvement of Src Family Tyrosine Kinases in Lymphangiogenesis

2006	Second European Conference on Tumor Angiogenesis and Antiangiogenic Therapy, September, Munich, Germany Čamaj, P., Brückel, M., De Toni, E., **Ischenko , I**., Guba, M., Jauch, K-W., Bruns, CJ. The interaction of apoptosis and angiogenesis in a tumor cell enviroment
2006	Second European Conference on Tumor Angiogenesis and Antiangiogenic Therapy, September, Munich, Germany Albrecht, U., **Ischenko, I**., Schwarz, B., Schrepfer, S., Schmid, G., Baumann, C., Guba, M., Heeschen, C., Jauch, K-W., Bruns, CJ. Efficacy of CXCR2 Inhibition on Tumor Angiogenesis in Pancreatic Cancer
2006	Second European Conference on Tumor Angiogenesis and Antiangiogenic Therapy, September, Munich, Germany Schmid, G., Huber, S., **Ischenko, I**., Guba, M., Joka, M., Schröferl, S., Bruns, CJ., Heeschen, C., Jauch, K-W., Graeb, C. Impact of FTY720 on Lymph- and Hemangiogenesis

Oral presentations:

2006 Deutsche Krebskongress, 22-26 March, Berlin, Germany

Ischenko, I., Čamaj, P., De Toni, E., Heeschen, C., Jauch, C-W., Bruns, CJ. The Effect of Src Kinase Inhibition on 5-Fluorouracil Chemosensitivity Is Related to Thymidylate Synthase Expression in Human Pancreatic Carcinoma Cells

2006 Kongress der Deutschen Gesellschaft für Chirurgie, 2-5 Mai, Berlin, Germany

Ischenko, I., Čamaj, P., De Toni, E., Heeschen, C., Jauch, C-W., Bruns, CJ. The Effect of Src Kinase Inhibition on 5-Fluorouracil Chemosensitivity Is Related to Thymidylate Synthase Expression in Human Pancreatic Carcinoma Cells

Chapter 13

OWN PUBLICATIONS

Conrad, C.,*** Ischenko I.,*** Köhl, G., Wiegand, U., Guba, M., Yezhelyev, M., Ryan, AJ., Barge, A., Geissler, EK., Wedge SR., Jauch, K-W., Bruns, CJ. Antiangiogenic and antitumor activity of a novel VEGFR-2 tyrosine kinase inhibitor ZD6474 in a metastatic human pancreatic tumor model. Manuscript accepted for the publication in "Anticancer Drugs", 2007

Ischenko, I., Schmid, G., Huber, S., Guba, M., Jauch, K-W., Bruns, CJ. Involvement of Src Family Tyrosine Kinases in Lymphangiogenesis. Angiogenesis 2006 (manuscript in preparation)

Ischenko, I., Čamaj, P., Guba, M., Graeb, C., Jauch, K-W., Bruns, CJ. The Effect of Src Kinase Inhibition on 5-Fluorouracil Chemosensitivity Is Related to Thymidylate Synthase Expression in Human Pancreatic Carcinoma Cells. Clin Can Res 2006 (manuscript in preparation)

Ischenko, I., Yezhelyev, M., Guba, M., Papyan, A., Jauch, K-W., Bruns, CJ. Effect of Src Kinase Inhibition on Metastasis and Tumor Angiogenesis in Human Pancreatic Cancer. Angiogenesis 2006 (manuscript submitted)

Čamaj, P., Ziegelaar, B., **Ischenko, I**., Schmid, G., Jauch, K-W., Bruns, CJ. Antiangiogenic effect of primary human chondrocytes. Angiogenesis 2006 (manuscript submitted)

Schmid, G., Guba, M., **Ischenko, I**., Brückel, M., Bruns, CJ., Jauch, K-W., Graeb, C. Administration of FTY720 Inhibits Angiogenesis and reduces Tumor Growth. Transplant Proc, 2006

Guba, M., Yezhelyev, M., Eichhorn, ME., Schmid, G., **Ischenko, I**., Papyan, A., Graeb, C., Seeliger, H., Geissler, EK., Jauch, K-W., Bruns, CJ. Rapamycin Induces Tumor-specific Thrombosis via Tissue Factor in the Presence of VEGF. Blood 2005

Bruell, D., Bruns, CJ., Yezhelyev, M., Huhn, M., Muller, J., **Ischenko, I**., Fischer, R., Finnern, R., Jauch, K-W., Barth, S. Recombinant Anti-EGFR Immunotoxin 425(scFv)-ETA Demonstrates Anti-tumor Activity Against Disseminated Human Pancreatic Cancer. Int J Mol Med 2005

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