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Receptor Cross-Talk in the Biology & Therapeutics Of Pediatric Rhabdoid Brain Tumors

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Receptor Cross-Talk in the Biology & Therapeutics Of Pediatric Rhabdoid Brain Tumors

By

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Abstract

Atypical teratoid rhabdoid tumor **(ATRT)** is a highly malignant brain tumor that usually affects very young children and typically causes death, despite very aggressive treatment. The biological properties contributing to tumor aggressiveness and resistance to common chemotherapeutic agents are currently unknown. Previous studies have shown the activation of Insulin like growth factor-I receptor **(IGF-1R)** in ATRT tumor specimens and cell lines. Additionally, angiogenesis is an established physiological mechanism that supports the survival and progression of brain tumors. Vascular endothelial growth factor receptor **(VEGFR)** signaling pathway is a major regulator of angiogenesis in brain tumors. We hypothesized that molecular interactions may exist between these two signaling pathways. Our findings show evidence for a novel IGF-1R/VEGFR-2 cross-talk in response to IGF-I mediated activation. Furthermore, we show evidence that the inhibition of IGF-1R/VEGFR-2 pathways by the small molecule inhibitors lead inhibition of cell migration properties and the initiation of apoptosis. Overall, the data generated in this set of studies present a framework to evaluate and utilize the receptor cross talk pathways to identify novel treatment approaches for ATRT in the future.

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I dedicate this thesis to
My family and my husband Essa,
For their constant support and unconditional love.
I love you all dearly.

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
AKT	Protein kinase B
ALS	Acid labile subunit
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
ATRT	Atypical Teratoid Rhabdoid Tumor
BAD	Bcl-2 associated death protein
BRCA1	Breast cancer1 tumor suppressor gene
cdc42	Cell division control protein 42 homolog
CI	Combination index
c-Myc	Cellular homolog of myleocytomatosis oncogene
CNS	Central Nervous System
COSMIC	Catalogue of Somatic Mutations in Cancer
CREB	Response Element Binding Factor
DAG	Diacylglycerol
DFO	Deferoxamine
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
EDTA	Ethylendiaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ErbB4	Erythroblastic leukemia viral homolog-4
ERK	Extracellular regulated kinase
ELISA	Enzyme linked immunosorbent assay
ELK-1	ETS-E-twenty six family of transcription factors
eNOS	Endothelial nitric oxide synthase
FAK	Focal-adhesion kinase
FBS	Fetal Bovine Serum
FGF	Fibroblast growth factor

FKHR	Forkhead-related transcription factor
Flk-1	Fetal liver kinase 1
Flt-1	Fms-like tyrosine kinase 1
Flt-4	Fms-related tyrosine kinase4
GDP	Guanine diphoshate
GH	Growth hormone
Grb2	Growth factor receptor-bound protein2
GSK	Glycogen synthase kinase
GTP	Guanine triphosphate
HBx	Hepatitis B Virus oncoprotein
HER2	Human epidermal growth factor receptor 2
HIF	Hypoxia-inducible factor
H-Ras	Harvey rat sarcoma viral oncogene homolog
HRP	Horseradish peroxidise
hsNF5	Human non sucrose fermenting 5
IC50	Half maximal inhibitory concentration
IGF	Insulin-like growth factor
IGF-I	Insulin-like growth factor-I
IGF-II	Insulin-like growth factor-II
IGFBP	Insulin-like growth factor binding protein
IGF-IR	Insulin-Like Growth Factor-I Receptor
INI1	Integrase interactor 1
IP3	Inositol-1, 4, 5-triphosphate
IQGAP1	Ras GTPase-activating-like protein
IR	Insulin-receptor
IRS	Insulin receptor substrate
JAK	Janus Kinase
JNK	Jun N-terminal kinase
KDR	kinase insert protein domain receptor
MAPK	Mitogen activated kinase
mg	milligram

ml	millilitre
μM	micro molar
mTOR	Mammalian target of rapamycin
Nck	Non catalytic region of TK adaptor protein1
NFkB	Nuclear factor kappa light chain enhancer of activated B cells
ng	nanogram
nM	nanomolar
NO	Nitric oxide
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor
PDK-1	Phosphoinositide-dependent protein kinase
pg	Picogram
PI3K	Phosphotidylinsitol-3kinase
PIP2	Phosphatidylinositol-4, 5-bisphosphate
PKC	Protein kinase C
PLC-γ	Phospholipase C-γ
Rac1	Ras-related C3 botulinum toxin substrate 1
Raf-1	Cellular homolog of viral raf gene v-raf
RTKs	Receptor tyrosine kinases
Rho	Ras homolg
SCF	Stem cell factor
Sck	Shc-related adaptor protein k
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH2	Src homology 2
Shb	SH2 domain-containing protein B
Shc	Src homology 2
siRNA	Small interfering RNA
SMARCB1	SWI/SNF related matrix associate regulator of chromatin subfamily B member1

SOS	Son of sevenless
Src	Sarcoma proto-oncogene
STAT	Signal transducer and activator of transcription
SWI/SNF	Switch/Sucrose Non Fermentable
SV40T	Simian Virus 40T antigen
Syp	Major synaptic vesicle protein p38
TGF	Transforming growth factor
TK	Tyrosine kinase
VEGF	Vascular endothelial growth factor
VRAP	VEGF receptor-associated protein
WT1	Wilms tumor protein 1

Chapter One: Introduction

1.1 Childhood Brain Tumors

Brain tumors are the most common solid tumors of childhood and the second most common form of cancer after hematological malignancies accounting for approximately 20 to 25% of all primary pediatric tumors [1]. Although considerable progress has been made in the outcomes of certain tumors such as medulloblastoma and low-grade glioma in which 5-year survival rates exceed 75%. However, prognosis of other types of childhood brain tumor remains poor despite the use of various strategies to intensify conventional chemotherapy and irradiation. Because the developing brain is highly vulnerable to treatment-induced cognitive and endocrine sequelae, particularly from radiotherapy, ongoing studies are exploring the use of intensive therapy or novel, molecularly targeted approaches not only to improve disease control rates but also the quality of survival in affected patients.

1.2 Atypical Teratoid Rhabdoid Tumor (ATRT)

Atypical Teratoid Rhabdoid Tumor is a highly malignant, central nervous system tumor that primarily occurs in very young children. Atypical Teratoid Rhabdoid Tumor accounts for 1-2% of central nervous system **(CNS)** tumors in children of all ages, but for10-20% of tumors in patients less than 3 years old [2]. This tumor, first described in 1987 by Rorke and Colleagues, was often classified as a medulloblastoma, primitive neuroectodermal tumor, or choroid plexus carcinoma prior to its recognition as a separate entity because they share indistinguishable gross, radiographic, and histopathological

features [3]. The World Health Organization (WHO) began classifying ATRT as an embryonal grade IV neoplasm in 1993 [4]. Because ATRT is a highly malignant tumor, patients typically have a fairly short history of progressive symptoms measured in days to weeks. Data from the ATRT registry suggest that approximately 20% of patients present with disseminated disease [5].

1.2.1 Pathologic and biologic features of ATRT

Atypical Teratoid Rhabdoid Tumor is morphologically heterogeneous because it contains highly cellular sheets of undifferentiated rhabdoid cells against a background of primitive neuroectodermal cells, mesenchymal cells, and epithelial cells. Apoptotic bodies, evidence for mitosis, folds of necrosis and dystrophic calcifications are usually seen. Expression of epithelial membrane antigen, vimentin, and smooth muscle actin are characteristic of these tumors [6]. Atypical Teratoid Rhabdoid Tumor is frequently associated with mutation or deletion of the human non-sucrose fermenting 5 ***(hSNF5)***/the integrase interactor 1 ***(INI1)***/ Switch/Sucrose Non Fermentable ***(SWI/SNF)*** related matrix associate dependent regulator of chromatin subfamily B member1 ***(SMARCB1)*** gene, found on chromosome 22q11.2. Switch/Sucrose Non Fermentable ***(SWI/SNF)*** related matrix associate dependent regulator of chromatin subfamily B member1 ***(SMARCB1)*** gene is a key component of the chromatin-remodelling complex, and can function as a tumor suppressor, but mutations can be identified in only 76% of CNS ATRT tumor samples. A small subset of ATRT patients exhibits germline mutations in the ***SMARCB1*** gene and subsequent somatic inactivation of the remaining copy of the gene, resulting in malignant transformation [7].

1.2.2 Treatment and Prognosis

There is currently no established standard curative treatment available for children with CNS ATRT. The majority of published data on outcome for ATRT is based on small, retrospective series, including patients treated with multiple therapeutic approaches, making standardization of therapy difficult. Given the highly aggressive nature of the tumor, most patients have been treated with intensive multimodal therapies, including intensive alkylating agents, as well as high-dose chemotherapy with stem cell rescue. Radiation is an effective mode of therapy, but it is often avoided in patients younger than 3 years old due to long- term neurocognitive complications. Data from the ATRT Registry suggests that patients who have had a complete resection may have a longer median survival, although complete surgical resection is often difficult, given the invasive nature of the tumor. Atypical Teratoid Rhabdoid Tumor is a deadly disease, with initial retrospective studies reporting a time course from diagnosis to death of about 12 months with standard therapy [2]. Current therapy is reaching the maximum levels of tolerable intensification without significantly changing outcomes. Hence the development of effective new therapeutic approaches is desperately needed to improve current outcome rates. This necessitates defining the key pathways and growth mechanisms of ATRT to identify potential therapeutic targets.

1.2.3 Mechanism of ATRT development

Data from histo-immunologic studies of ATRT suggest that the hallmark rhabdoid cells may constitute a highly malignant, tumor stem cell population that arises from transformation of a multipotential progenitor cell with marked proliferative and invasive features. The genomic lesion that defines ATRT, and that presumptively initiates the process of malignant transformation, is the homozygous inactivation of the *INI1* gene

located on chromosome band 22q11.2. The *INI1* gene alterations appears to affect cell-signalling by its ability to interact with key signalling molecules and tumor suppressors, in order to modify the response to factors mediating cell differentiation programs [8]. For example, it has been shown that *INI1* plays a critical role in interferon signalling and interferon stimulated gene expression patterns [9]. The role of growth stimulatory molecules and pathways was demonstrated by a screening of receptor tyrosine kinases, which identified the presence of phosphorylated erythroblastic leukemia viral oncogenehomolog-4 **(ErbB4)**, Insulin-receptor **(IR)**, platelet derived growth factor receptor **(PDGFR)** and IGF-I R in ATRT cell lines [10]. The activities of these receptors have been implicated in the tumorigenicity, proliferation, metastasis and the development of drug resistance. These data can be effectively utilized to study the biology of these cells and to identify targets for potential therapeutic agents.

1.3 Receptor Tyrosine Kinases

Receptor tyrosine kinases **(RTKs)** belong to the class of transmembrane receptors with intrinsic protein-tyrosine kinase activity. Receptor tyrosine kinases transmit key regulatory signals involved in cell proliferation and differentiation, cell survival and metabolism, cell migration, and cell cycle control [11]. All RTKs have a similar molecular architecture, with ligand-binding domains in the extracellular region, a single transmembrane helix, a cytoplasmic region that contains the protein tyrosine kinase **(TK)** domain and additional carboxy (C-) terminal and juxtamembrane regulatory regions. Approximately 20 distinct human-RTK subfamilies have been identified to date that display structural varieties in their extracellular domains, like cysteine rich domains, leucine rich domains or immunoglobulin rich domains. The members of a given subfamily

are similar in structure but differ in tissue and ligand specifity. Figure 1 shows an overview of the human receptor tyrosine kinases.

Receptor tyrosine kinase signalling requires ligand-induced receptor dimerization which results in the trans-phosphorylation of tyrosine residues in the dimeric receptor subunits; a process called auto-phosphorylation. The phosphorylated residues function as docking sites for numerous adaptor proteins. These in turn can activate a number of signalling cascades, including the mitogen activated kinase **(MAPK),** phosphotidylinsitol-3kinase **(PI3K)**, Janus Kinase **(JAK)**, phospholipase C-γ **(PLC-γ)** and Jun N-terminal kinase **(JNK)** signalling pathway [12]. Mutations in RTKs and aberrant activation of their intracellular signalling pathways have been causally linked to cancers, diabetes, inflammation, severe bone disorders, arteriosclerosis and angiogenesis. The aberrant activation of RTKs is mediated by four principal mechanisms: autocrine activation, chromosomal translocations, RTK over-expression, or gain-of-function mutations [11]. Recent sequencing efforts in a wide variety of tumors have identified mutations in numerous RTKs [Catalogue of Somatic Mutations in Cancer **(COSMIC)** database, 13].

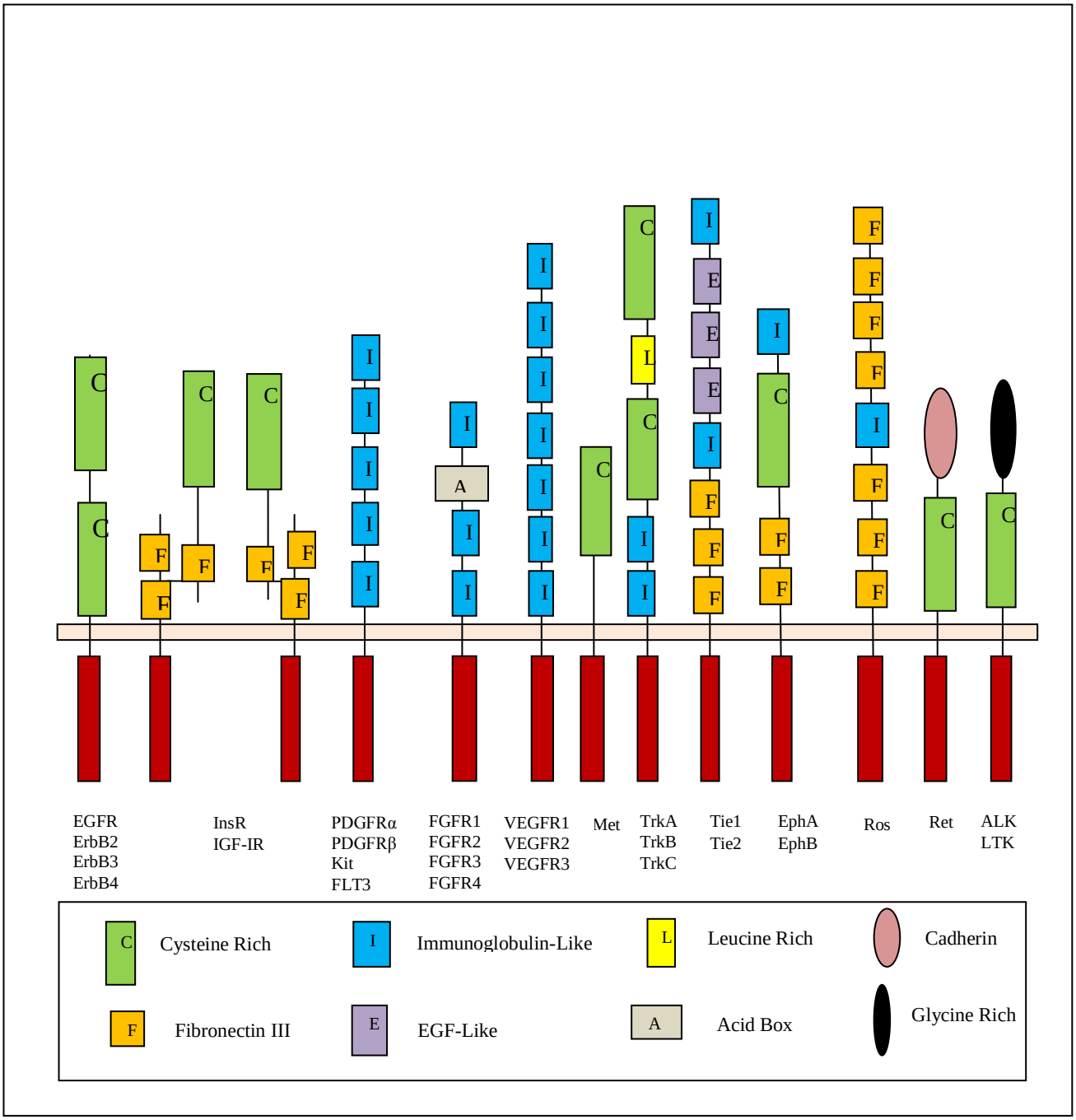


Figure1.1 Human receptor tyrosine kinases.

Diagrammatic representation of several RTK family members. Members of each receptor sub-family are indicated below each RTK. Receptor tyrosine kinases structural domains are identified according to the key box.

1.4 The Insulin-like Growth Factor (IGF) system

The IGF system is a complex signalling network that includes multiple ligands, receptors and regulatory proteins. It has been proven to play an integral role in growth, differentiation, and developmental processes, and is also involved in many physiological and pathological processes, such as mitogenesis, differentiation, tissue homeostasis, anti-apoptosis and cell motility. It is activated during fetal development with growth-promoting effects but it is also implicated in postnatal growth and tissue remodelling [14]. Dysregulation of IGF system expression and action is linked to diverse pathologies, ranging from growth deficits to cancer development. Over the past two decades research has shown the importance of the IGF axis in tumorigenesis, metastasis and resistance to existing forms of cancer therapy [15].

1.4.1 IGF growth factors and binding proteins

The IGF system has two key circulating ligands of the insulin-related peptide family; the insulin-like growth factor-I **(IGF-I)** and the insulin-like growth factor-II **(IGF-II)**. The human *IGF-I* gene is located on chromosome 12 [16]. The mature IGF-I is a single chain, 7.5KDa, 70-amino acid peptide cross linked by 3 disulfide bridges [17]. The IGF-I protein is produced primarily by the liver in response to growth hormone **(GH)** secretion by the anterior pituitary gland. Binding of GH to its receptor in the liver results in IGF-I synthesis and secretion. In addition, insulin can indirectly increase IGF-I production by up regulating GH receptors [18]. IGF-I is also synthesized in other organs independently of GH regulation, where it exerts autocrine or paracrine effects [18]. High affinity binding of IGF-I to IGF-IR leads to the initiation of the subsequent physiological response. Similar to IGF-I, IGF-II is secreted both in the liver and in extrahepatic tissues but this process is not regulated by GH. The human *IGF-II* gene is located on chromosome 11[16].The structure

of IGF-II resembles that of IGF-I but consists of a shorter peptide with67 amino acids [17]. IGF-II has high affinity for IGF-IIR, which does not transduce a signal; rather it exerts antiproliferative and proapoptotic activities by limiting IGF-II bioavailability and thereby reducing its interaction with the IGF-IR [19].

In circulation, both of these ligands exist as complexes with one of six insulin-like growth factor binding proteins **(IGFBPs**, IGFBP-1 to IGFBP-6). IGFBPs have higher affinity for IGFs than their cognate receptors; therefore IGFBPs stabilize, prolong the half life of IGFs, and consequently modulate their bioavailability and activity [20]. IGFBPs can be produced by the liver and function as endocrine factors or they can be produced by non-hepatic tissues and operate in an autocrine or paracrine manner. Several factors have been shown to increase IGFBPs synthesis, including estrogens, retinoids, and vitamin D [21-23]. IGFBPs differ in structure, binding characteristics, and function in a complex manner (table1.1). More than 75% of IGF-I is confined to the vascular compartment as a complex with IGFBP-3, the most abundant circulating IGFBP and a non IGF binding component named acid labile subunit **(ALS)** [24]. Some IGFBPs display IGF-independent actions. For example IGFBP-3 has both growth-inhibiting and growth-promoting effects at the cellular level, which can be either dependent or independent of IGFs [25]. The complex molecular mechanisms involved in the IGFBP regulation of IGF-I and IGF-II are affected by numerous factors, such as IGFBP expression levels, tissue distribution, phosphorylation, proteolysis and cell surface association.

IGFBP	IGFBP characteristics
IGFBP-1	-Physiological levels stimulate IGF-I action -Molar excess inhibits mitogenic and insulin-like effects of IGF-I and IGF-II
IGFBP-2	-Inhibits IGF- induced DNA synthesis -Observed to potentiate IGF function
IGFBP-3	-Major carrier of IGF-I and IGF-II in serum -Modulate IGF endocrine function -Potentiates IGF activity -Excess levels are inhibitory
IGFBP-4	-Consistently inhibits IGF action -Serum concentration is generally low -Tissue-specific expression
IGFBP-5	-Inhibitory action -Association with extracellular matrix lowers affinity for the IGFs thereby increasing IGF activity
IGFBP-6	-Specifically binds IGF-II -Generally thought to be inhibitory

Table 1.1 Insulin-like binding protein functions.

The major functions and characteristics are presented for each individual IGFBP

1.4.2 Biological functions of IGF-I and IGF-II

The IGFs are the key regulators of cell growth and differentiation in many tissues. They have properties of tissue growth factors, but also have an additional well recognized function as hormones that regulate growth and energy metabolism. They are involved in pleiotropic actions at endocrine, paracrine and autocrine levels. For example, IGFs promote skeletal muscle regeneration and hypertrophy [26], as well as mammary gland cell proliferation during puberty, pregnancy and lactation [27].

Insulin-like Growth Factors also have a protective function in the heart. Low serum IGF-I levels are associated with increased risk of ischemic heart disease and stroke [28]. In addition IGF-I plays an important neuroprotective role by supporting neuronal development, metabolism, survival and regeneration [29].

At the cellular level, IGF-I is required for cell cycle progression from G1 to S (DNA synthesis) phase. [15]. In some cells, IGFs proliferative effects may lead to induction of differentiation. At the end of the M phase, the daughter cells may re-enter the cell cycle or undergo terminal differentiation. IGF-I has been demonstrated to induce differentiation of myoblasts, osteoblasts, adipocytes, oligodendrocytes, neurones, and hematopoietic cells [30].

Besides its role in somatic growth, IGF-I has been shown to exert metabolic effects in its target cells. For example, IGF-I mediates the anabolic effects of GH by stimulating protein synthesis and preventing proteolysis [31]. IGF-I has been used clinically to treat certain catabolic illnesses [32]. IGF-I potently stimulates peripheral tissue glucose uptake and glycogen synthesis [31]. Individuals with *IGF-I* gene deletion have low levels of circulating IGF-I and increased insulin resistance [33]. Treatment with recombinant IGF-I

in these conditions, and in patients with type I and type II diabetes, has been shown to improve insulin sensitivity and glucose homeostasis [34-35].

The activity of IGF-II appears to be similar to that of IGF-I. IGF-II appears to be more important in embryonic and fetal growth as it is expressed throughout life where its activity is regulated by genomic imprinting [36].

1.4.3 The Insulin-Like Growth Factor-I Receptor (IGF-I R)

Insulin-Like Growth Factor-I Receptor is a heterotetrameric complex comprised of two α- and two β-subunits. Each α-subunit contains 706 amino acids, and each β-subunit contains 627 amino acids. One α-subunit and one β-subunit are linked by a disulfide bond to form an α/β-half receptor, which, in turn, is linked to another α/β-half receptor by two disulfide bonds between the two α-subunits to form the mature, functional receptor. The α-subunit is localized entirely extracellularly and contains a cysteine-rich domain, which is necessary for high-affinity IGF-I binding. The β-subunit spans the membrane and is localized primarily intracellularly. The intracellular portion contains a tyrosine kinase domain, whose activation is crucial for the propagation of IGF-I effects [37]. For a schematic overview of IGF-1R please see figure 1.2.

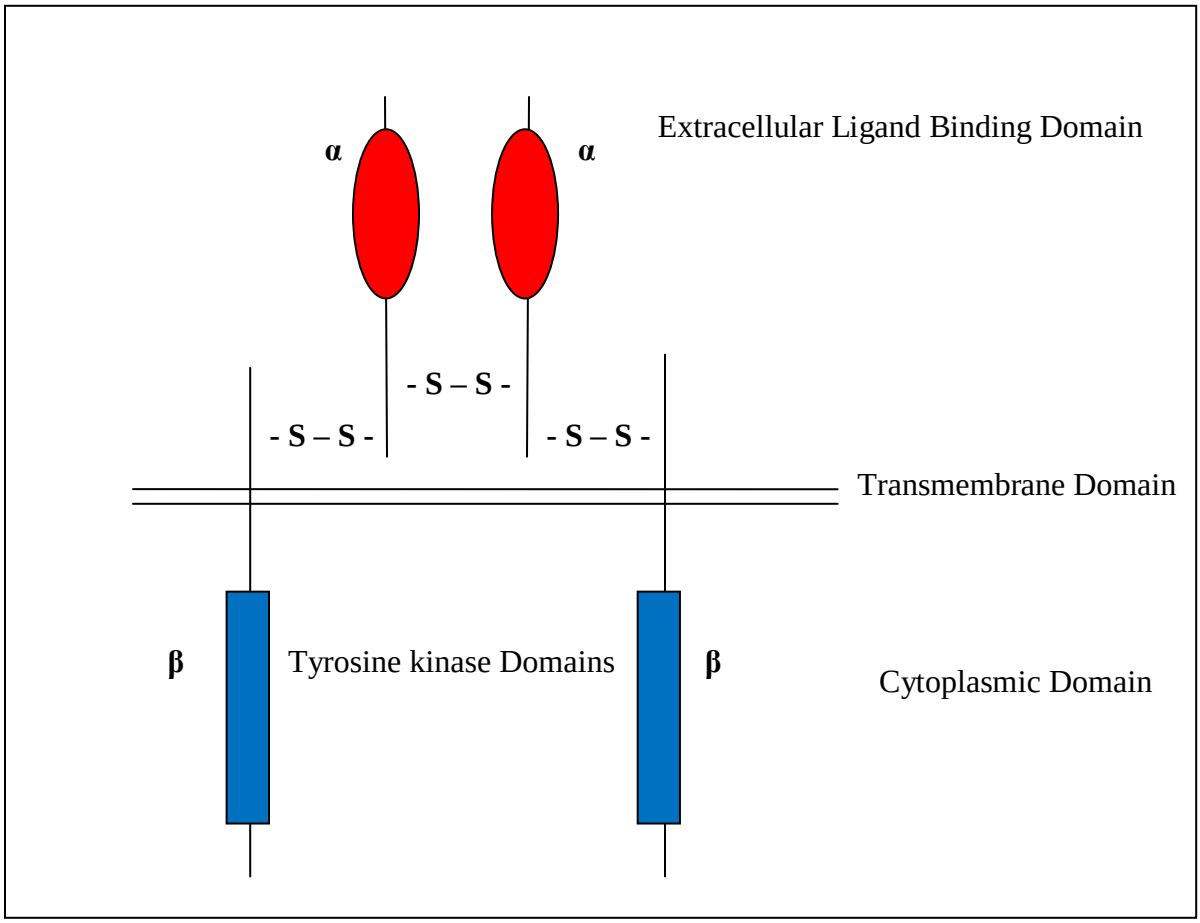


Figure 1.2 Schematic diagram of the IGF-IR structure.

1.4.4 IGF-IR signalling

Insulin-Like Growth Factor-I Receptor binds to IGF-I and IGF-II with high affinity and to insulin with very low affinity. Following ligand binding to the α-subunit, the three tyrosine residues (Tyr 1131, Tyr1135, and Tyr 1136) of the activation-loop within the tyrosine kinase domain of the β-subunit are trans-autophosphorylated[38]. In addition, phosphorylation of additional tyrosine residues in other areas of the β-subunit provides docking sites that allow for the recruitment of adaptor proteins. Phosphorylation of adaptor proteins leads to binding of additional proteins, allowing for signal transduction along several specific pathways [38]. Downstream signalling is mostly channelled through MAPK/extracellular regulated kinase **(ERK)** pathway, and PI3K/protein kinase B **(AKT)** / mammalian target of rapamycin **(mTOR)** pathway, leading to increased cell proliferation, and decreased apoptosis. Further important residues for the receptor function are Tyr950 which is the main binding site for the downstream signalling molecules including insulin receptor substrate family members **(IRS1-4)**, and src homology 2 **containing** transforming protein (Shc). Both are critical for proliferative and the transforming capacities of IGF-IR [39-40]. Several residues within the C-terminal domain have been found to be required for the anti-apoptotic, migration, and invasion properties of IGF-IR (Tyr 1250, and Tyr1251) [41].

1.4.4.1 PI3K Pathway

The activation of the PI3K pathway requires the binding of insulin receptor substrate proteins via their SH2 domains to Tyr950 in the juxtamembrane region. Upon binding, these proteins become tyrosine phosphorylated and subsequently evoke the binding of other signalling adaptor proteins like growth factor receptor-bound protein2 **(Grb2)**, non –catalytic region of tyrosine kinase adaptor protein1 **(Nck1)**, major synaptic

vesicle protein p38 **(Syp)**, and the regulatory subunit p85 of PI3K which binds to the catalytic subunit of PI3K. This leads to the recruitment of Akt to the membrane and allows the constitutively activated phosphoinositide-dependent protein kinase **(PDK-1)** to phosphorylate and activate Akt. The activated Akt then mediates the anti-apoptotic effects of the IGFs by phosphorylating and inhibiting several pro-apoptotic downstream targets like glycogen synthase kinase **(GSK3)**, Bcl-2 associated death protein **(BAD),** and caspase9, as well as transcription factors like cyclic adenosine monophosphate **(AMP)** Response Element Binding Factor **(CREB)** and the forkhead-related transcription factor **(FKHR)** family. Furthermore, Akt participates in the activation of nuclear factor kappa-light-chain-enhancer of activated B cells **(NFkB)** and mTOR, resulting in the transcription of genes which mediates cell survival and protein synthesis [42].

1.4.4.2 MAPK pathway

The recruitment of Shc to Tyr950 of the activated IGF-IR stimulates the Ras/Raf/MAPK pathway [43]. Src homology 2 serves as an adaptor for the SH2-domain containing signalling molecule Grb2. Grb2 is bound to the guanine nucleotide releasing factor son-of -sevenless **(SOS)** via its SH3 domains, bringing it closely to the guanine triphosphatase **(GTPase).** Ras becomes activated through the exchange of guanine diphoshate **(GDP)** to guanine triphosphate **(GTP)** and hereby binds and activates the serine/threonine kinase of cellular homolog of the viral raf gene, v-raf **(Raf-1)**. Activated Raf-1 phosphorylates the MAPK kinase MEK1/2 which phosphorylates the MAP kinase Erk1/2, thereby enabling it to translocate into the nucleus where it phosphorylates and activates a number of transcription factors such as STAT-1 STAT-3, ELK-1, and c-Myc [43]. Figure 1.3 represents the IGF-I activated MAPK/ERK and PI3K/Akt signalling cascades.

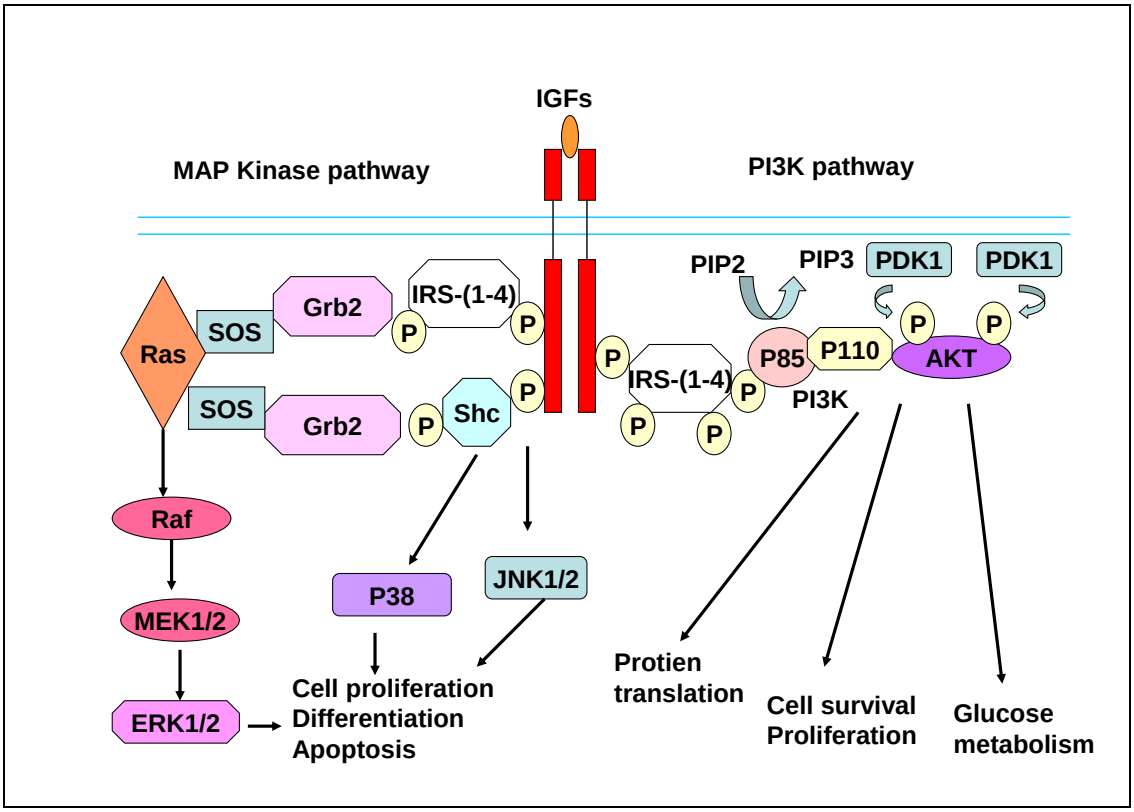


Figure 1.3 IGF-IR signal transduction pathways.

Ligand binding to the IGF-IR induces conformational change resulting in phosphorylation of the receptor tyrosine kinase domain and recruitment of the IRS and Shc substrates to the receptor. Binding of these substrates initiates signalling cascades which activate the PI3K and MAPK pathways regulating cell proliferation, apoptosis, growth, transcription, translation and metabolism.

1.5 The Role of IGF-IR axis in Cancer

Epidemiological and laboratory studies have provided strong evidence implicating the IGF system in numerous different cancers, including breast cancer, colorectal cancer, liver cancer, prostate cancer, pancreatic cancer, multiple myeloma, melanoma, glioblastoma, mesothelioma and childhood cancers [44]. Epidemiological studies have linked high circulating levels of IGF ligands and polymorphism in relevant genes to cancer risk and prognosis [45]. For example, a comprehensive meta-analysis of case-control studies indicated high circulatory concentrations of IGF-I were associated with increased risk of prostate, colorectal and premenopausal breast cancer [46].

1.5.1 IGF-IR and Oncogenes

The IGF-IR gene is constitutively expressed in most cells. The IGF-IR promoter exhibits a high basal transcriptional activity. The IGF-IR level is regulated by physiological conditions including nutritional factors, hormonal stimulation, the developmental stage and cellular factors including transcription factors, oncogenes and suppressor genes [47-49]. There is evidence suggesting an interplay between tumor suppressors and the IGF axis. Oncogenes, such as the Hepatitis B Virus oncoprotein **(HBx)** or Ewing Sarcoma fusion proteins, recruit and activate the IGF-IR signalling pathway by increasing transcription of the IGF-IR gene, while loss of tumour suppressor genes, such as p53, breast cancer 1 **(BRCA1)** or Wilms tumor protein **(WT1)**, results in IGF-IR over expression by loss of transcriptional control [50].

1.5.2 Transformation

The IGF-IR plays an important role in oncogenic transformation. This was first recognised in fibroblasts derived from homozygous IGF-IR null mice embryos. In the absence of IGF-IR, they become resistant to malignant transformation by a number of

oncogenes (e.g. Simian Virus 40T antigen **SV40T**, Ewing Sarcoma fusion protein). Re-expression of the IGF-IR restored susceptibility to transformation in these cells [51]. IGF-IR null cells are resistant to transformation by several oncogenes, including the SV40 T antigen, activated Harvey rat sarcoma viral oncogene homolog **(H-Ras)**, bovine papillomavirus E5 protein, human papillomavirus E7 protein, Ewing's sarcoma fusion protein, activated sarcoma proto-oncogene **(Src)** and others [52]. Cellular transformation and progression of several types of sarcoma, including rhabdomyosarcoma, synovial sarcoma, leiomyosarcoma, Ewing's sarcoma and osteosarcoma are influenced by IGF-1R [53].

1.5.3 Proliferation and anti-apoptosis

Insulin-Like Growth Factor-I Receptor mediated MAPK/PI3K activation has been proven to support cancer progression through enhancement of mitogenesis or suppression of apoptosis [54]. Promoting cell cycle and escaping from cell cycle arrest are the common characteristics of tumor cells. IGF-IR mediated MAPK promotes Cyclin D1 expression, while AKT activation prevents Cyclin D1 nuclear export and degradation by inhibiting GSK-3β activity [55]. This cell cycle progression in breast cancer cells can be reduced by PI3K inhibitors. Activity of c-myc, a transcription gene promoting survival, can be stimulated by activation of NF-kB followed by AKT activation [56].

1.5.4 Migration and metastasis

Cancer metastasis is composed of multiple processes, including tumor cell adhesion, migration, extracellular matrix **(ECM)** proteolysis and invasion. IGF-IR activation or over-expression has been shown to be associated with an increased propensity for invasion and metastasis. This is mediated by multiple signaling intermediates that influence invasive potential. IGF-induced phosphorylation of IRS-1 influences the

interaction between E-cadherin and β-catenin, enhancing β-catenin transcriptional activity and disconnecting E-cadherin from the actin cytoskeleton [57]. Similarly, tumor cell motility and invasive potential are influenced by crosstalk between the IGF axis and integrins [58], and by IGF-induced secretion of matrix metalloproteinases [59].

IGF-I is reported to induce angiogenesis by stimulating the migration and morphological differentiation of endothelial cells [60]. Another direct effect of IGF on angiogenesis is demonstrated in mice with vascular endothelial cells knockout IGF-IR or IR, in which remarkable reduction of retinal vessel formation is observed [61]. IGF-I and insulin are both involved in regulation of vascular endothelial growth factors **(VEGFs)** [62], by activation of AKT and MAPK signaling, leading to stabilization of hypoxia-inducible factors **(HIF-1α and HIF-2α)**, and up regulation of VEGF [63].

1.6 Tumor Angiogenesis

Angiogenesis is a physiological process involving the growth of new blood vessels from pre-existing vessels.Though angiogenesis normally occurs throughout life, in both health and disease, beginning in utero and continuing on through old age. However, tumors hijack this process in order to facilitate further growth and spread. In the late 1960s, the first preliminary evidence was presented indicating that tumor angiogenesis was mediated by diffusible factors produced by tumor cells [64]. Tumors less than 1 mm can usually obtain adequate nutrients from its surroundings by diffusion, but larger tumors require new blood vessels for support. [65]. Angiogenesis is considered one of the hallmarks of cancer development together with self-sufficiency in growth signals, tissue invasion and metastasis, insensitivity to anti-growth signals and evasion of apoptosis [66]. It is regulated by the highly coordinated function of various proteins with pro- and antiangiogenic functions. Proangiogenic factors include VEGF, fibroblast growth factor **(FGF)**, platelet-derived growth factor **(PDGF)**, IGF, transforming growth factor **(TGF)**, angiopoietins, and several chemokines; antiangiogenic factors include thrombospondin-1, angiostatin, and endostatin [66].

Hypoxia appears to be the major trigger for the initiation of angiogenesis. In this process, HIF-1α levels increase dramatically and modulate the levels of other molecules responsible for angiogenesis. Angiogenesis may be divided into four stages [67]:

(I) Activation of the endothelial cells leads to the localized degradation of the basal membrane of the parent vessel and of the surrounding ECM

(II) Oriented migration of endothelial cells in the ECM

(III) Proliferation of endothelial cells

(IV) Differentiation of these cells with organization into tubular structures with a new basal lamina.

Through these stages the new capillaries form a new vascular network.

1.7 Vascular endothelial growth factors and receptors

VEGFs and their receptors play a central role in the vasculature-related processes in the adult organism. The VEGF family comprises six secreted glycoproteins of which VEGF-A, VEGF-C, and VEGF-D are of great significance [68]. These VEGF ligands mediate their angiogenic effect via RTKs: VEGFR-1, also known as fms-like tyrosine kinase 1**(Flt-1)**, VEGFR-2, also known as kinase inserts protein domain receptor **(KDR)** or fetal liver kinase 1 **(Flk-1),** and VEGFR-3, also known as fms-related tyrosine kinase4 **(Flt-4)** [69-71]. VEGF RTKs are essential components of signal transduction pathways that affect cell proliferation, differentiation, migration, and metabolism. Activation of VEGF RTKs occurs through ligand binding, which facilitates receptor dimerization and autophosphorylation of tyrosine residues in the cytoplasmic portion (figure1.4). The phosphotyrosine residues either enhance receptor catalytic activity or provide docking sites for downstream signaling proteins [72].VEGF-A has been regarded as the major player for angiogenesis and usually referred to as VEGF. It binds to VEGFR-1 and VEGFR-2, but VEGFR-2 is the major mediator of the mitogenic and angiogenic effects of VEGF-A [73]. VEGF-C and VEGF-D activate VEGFR-3 and are important for lymphatic endothelial cell growth, migration and survival [74]. However, proteolytically processed VEGF-C and VEGF-D can also induce blood-vessel growth by activating VEGFR-2 [75]. VEGFR-3 deletion leads to defects in blood-vessel remodelling and embryonic death at mid-gestation. In addition blocking of VEGFR-3 suppresses angiogenic sprouting, indicating that activation of VEGFR-3 promotes angiogenesis in addition to lymphangiogenesis [76].

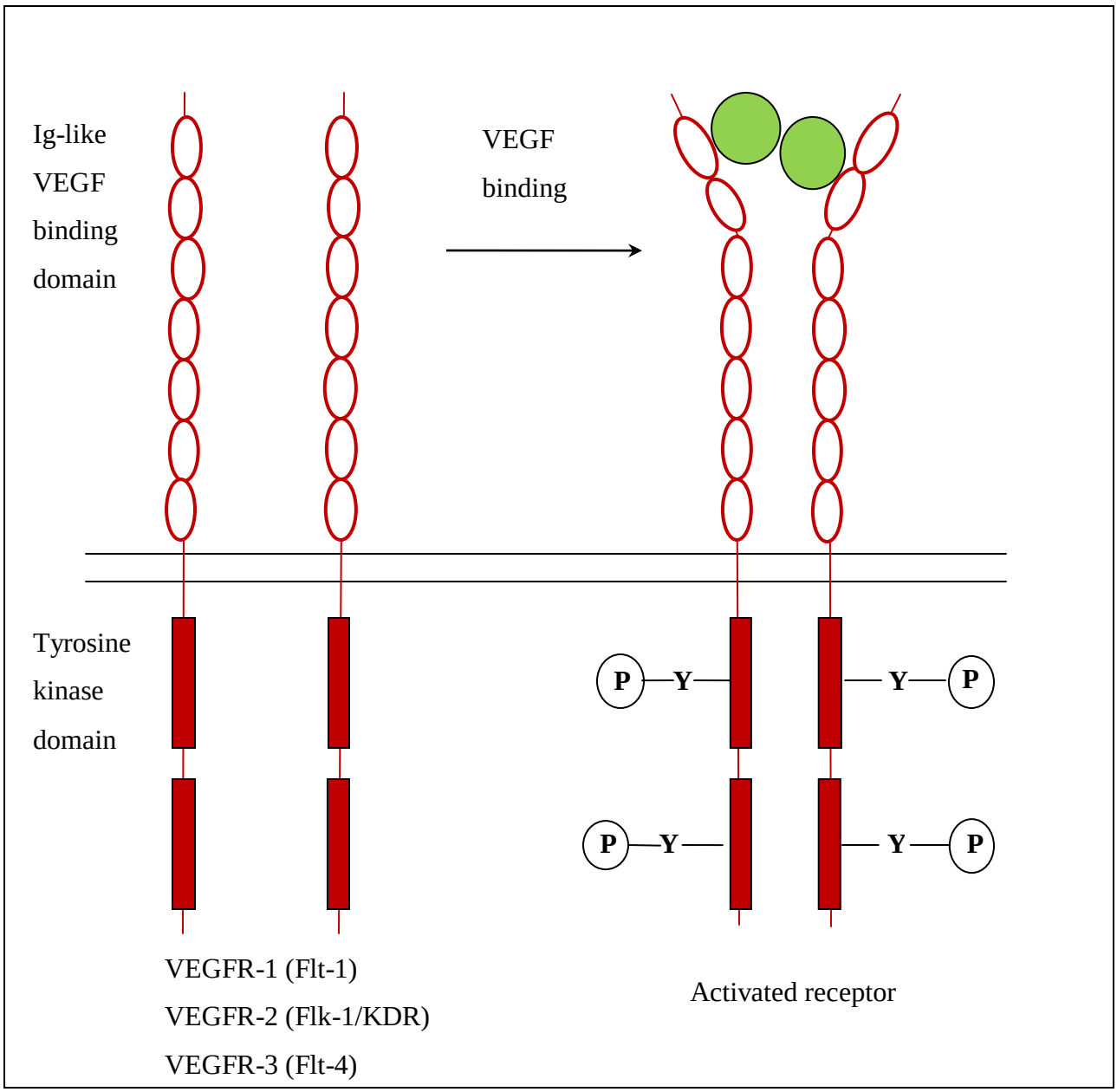


Figure 1.4 Representative structures of VEGF tyrosine kinase receptors.

The VEGF receptor family is represented by seven immunoglobulin-like loops in the extracellular domain, which binds VEGF. Two VEGF receptors form a dimer to activate autophosphorylation of tyrosine residues on the cytoplasmic domain. Ig = immunoglobulin; VEGF = vascular endothelial growth factor; Y- = phosphorylated tyrosine residues.

1.7.1 VEGFR-2 signalling

The critical role of VEGFR-2 in vascular development has been highlighted in the analysis of VEGFR-2 null mice. Loss of VEGFR-2 causes lethality of the mutant embryo, due to defects in the formation of blood islands, indicating that the receptor is required from the very first step of vascular development [77]. Complex cascades of signalling mechanisms operated downstream of VEGFR-2 serve to mediate the receptor stimulatory effects on a vast array of cellular activities ranging from cell proliferation, migration, survival, to vascular permeability (figure 1.5).

Cell Proliferation: Ligand binding triggers receptor dimerization and subsequent autophosphorylation of specific tyrosine residues in the receptors (figure1.5), which thereby serve as docking sites for the recruitment of Src homology 2 **(SH2)** domain containing proteins. One of the few SH2 domain containing proteins that has been shown to interact with VEGFR-2 is PLC-γ, which is recruited to the phosphorylated Tyrosine (Tyr) 1175 of VEGFR-2 [78]. Subsequent phosphorylation of PLC-γ gives rise to its active form, which mediates the hydrolysis of phosphatidylinositol-4, 5-bisphosphate **(PIP2)** to generate inositol-1, 4, 5-triphosphate **(IP3)** and diacylglycerol **(DAG)**. In turn, DAG and the increased intracellular calcium concentration by IP3 activate protein kinase C **(PKC)**. Finally, MAPK / ERK1/2 cascade, which is activated downstream of PKC, can induce endothelial cell proliferation [78].

Migration: Tyr1175 is a focal point of VEGFR-2 signalling. Mutation of the tyrosine residue at this specific site to phenylalanine causes embryonic lethality in mice, due to a severe defect in vascular formation [79]. In addition to PLC-γ, the SH2 domain-containing protein B **(Shb)** and the Shc-related adaptor protein **(Sck)** are recruited to VEGFR-2 by binding to Tyr1175 [80]. The PI3K mediated generation of

phosphatidylinositol-3, 4; 5-trisphosphate is known to activate the Ras homolg **(Rho)** family member of GTPases Ras-related C3 botulinum toxin substrate 1 **(Rac1)** which, in turn, triggers cell motility [81]. The actin binding protein Ras GTPase-activating-like protein **(IQGAP1)** can bind and activate Rac1 by inhibiting its intrinsic GTPase activity and thereby increases active (GTP-bound) Rac1. In accordance with this model, VEGF stimulation has been shown to promote the association of Rac1 and IQGAP1 complex to phosphorylated VEGFR-2, in order to facilitate endothelial cell migration in an in vitro setting [82]. Activation of PI3K results in creation of the membrane bound PIP3, as well as subsequent membrane targeting and activation of protein kinase B **(**PKB/Akt). Girdin (Girders of actin filament), an Akt substrate actin-binding protein, has recently been shown to play an important role in angiogenesis by facilitating endothelial cell migration [83].

Focal-adhesion kinase **(FAK)** was also reported to bind to Shb. A signalling pathway involving VEGF-induced FAK phosphorylation and recruitment of its substrate, the actin anchoring protein paxillin, might also be involved in VEGFR-2 mediated cell migration [84].

Two other phosphorylation sites, Tyr951 and Tyr1214, might also be involved in VEGF-mediated cell migration. Tyr951 serves as a binding site for VEGF receptor-associated protein **(VRAP)**, also known as Tsad, T-cell specific adaptor [85]. Phosphorylated Tyr1214 was reported to associate with the adaptor protein Nck (non-catalytic region of tyrosine kinase adaptor protein), which facilitate actin remodelling through recruitment of the Src family protein Fyn, in order to activate cell division control protein 42 homolog **(cdc42)** and MAPK [86].

Vascular permeability: Generation of nitric oxide **(NO)** by endothelial nitric oxide synthase **(eNOS)** has been shown to be essential for VEGF induction of vascular

permeability [87]. Activation of eNOS requires Akt-dependent phosphorylation [88-89]. The endothelial cell specific adhesion molecule VE-Cadherin is the key component of endothelium adherens junction that plays a main role in the control of vascular integrity and permeability [90]. The tyrosine kinase Src recruited to VEGFR-2, appears to be responsible for the disruption of VEGFR-2/VE-Cadherin complex, and increased vascular permeability upon VEGF stimulation [91-92]. Serine phosphorylation of VE-Cadherin by p21-activated kinase promotes its internalization into clathrin-coated vesicles and thereby triggers the disruption of intercellular junctions [93].

Survival: Akt signalling downstream of PI3K has been shown to be essential for endothelial cell survival [94]. This might involve Akt-mediated phosphorylation of apoptotic proteins BAD and caspase 9, which inhibits their apoptotic activities and thereby promotes cell survival [95].

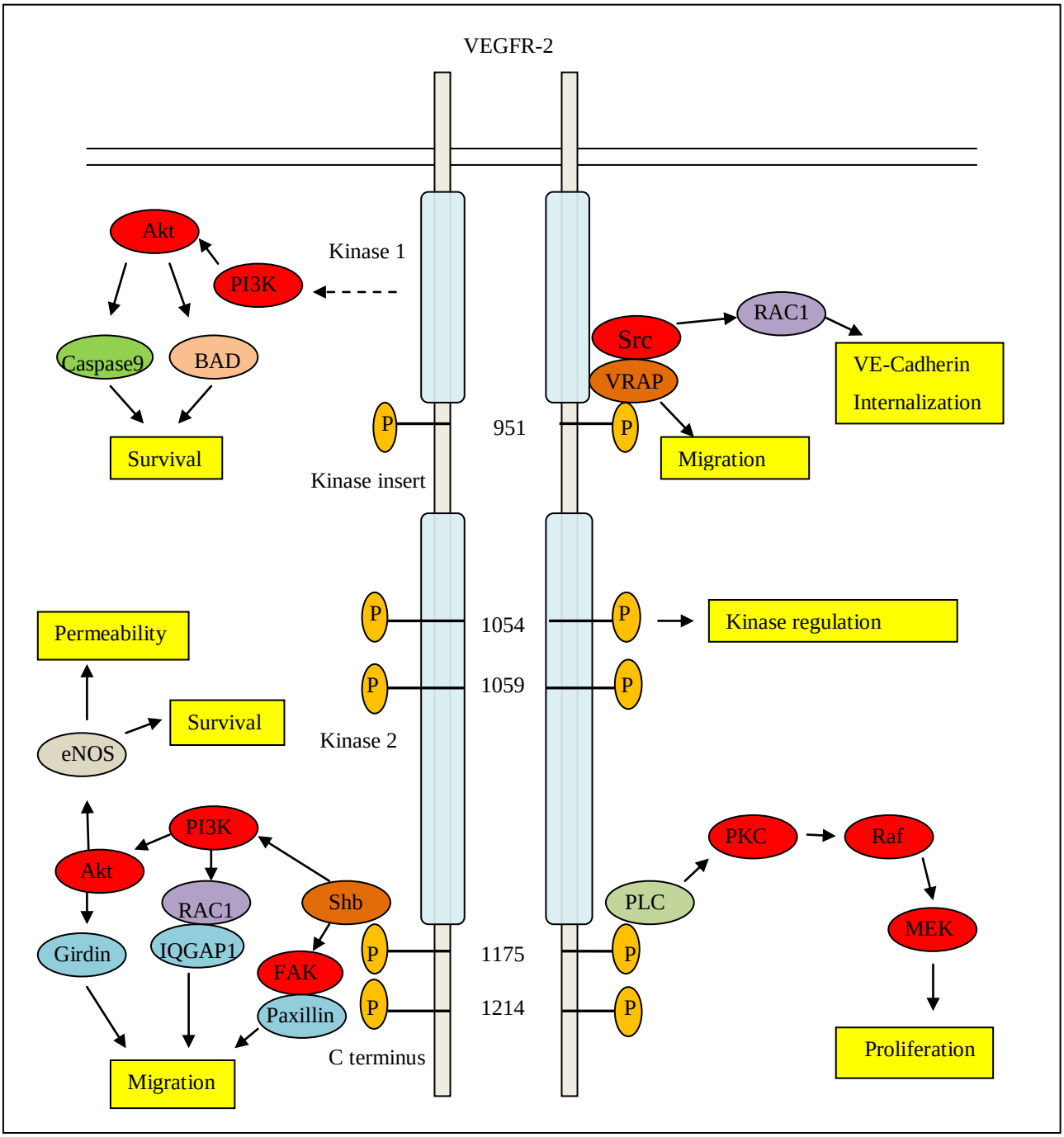


Figure 1.5 Schematic representations of VEGFR-2 signalling pathways.

Binding of VEGF to the VEGFR-2 extracellular domain causes receptor homodimerization, resulting in autophosphorylation of the RTK domain, leading to signalling events that regulate endothelial cell proliferation, migration, survival, and permeability.

1.8 Receptor cross-talk

Signalling cross-talk is characterized by the influence of one receptor/signalling system on a separate receptor/signalling system, leading to activation of multiple responses in the cell. This is an important subject with crucial implications in the efficacy of novel therapeutics.

Cancer cells are extremely adaptable or plastic in their ability to utilise cellular pathways that control the aspects of their physiology. For example, through over expression of a particular receptor they may have apparently adopted a dominant growth regulatory pathway. However, evidence is accumulating to demonstrate that they can readily switch to using alternative signalling pathways to maintain cell survival when this dominant pathway is blocked, a process paradoxically that in many instances is induced by the anti-cancer drugs themselves, thus limiting their activity and promoting resistance. This applies to IGF-IR and its cross-talk was described in different biological systems and may take place at variety of levels.

Interconnections were identified with nuclear steroids receptors [96], G protein coupled-receptors [97], and TGFβ signalling pathway [98]. Most studies reported the IGF-IR cross-talk occurring within the RTK family. Previous studies have shown that ligand stimulated IGF-IR shares the Ras pathway with other RTKs such as the epidermal growth factor receptor **(EGFR)** and PDGFR [99-100].

In glioblastoma cells, studies showed a compensatory up regulation of IGF-IR level in response to EGFR inhibitory treatment[101], and the impairment of IGF-IR function increased the apoptotic effects of EGFR inhibition[102].The IGF-I/IGF-IR pathway transactivated the EGFR via an autocrine release of EGF-like growth factors [103]. In mammary epithelial cells, IGF-IR signalling protected against apoptosis via EGFR

transactivation [104]. In NSCLC cells, amphiregulin transactivated IGF-IR independent of binding to its specific receptor EGFR [105].

In breast cancer cell models that over expressed human epidermal growth factor receptor 2 (HER2), an increased level of IGF-IR signalling found to interfere with Trastuzumab-induced HER2 inhibition [106]. Other experiments performed on mammary tumors demonstrated the existence of a hierarchal interaction between IGF-IR and HER2, in which IGF-IR directed HER2 phosphorylation. The physical association of both receptors, resulting in the formation of a heteromeric complex has been suggested to be the underlying reason for this interaction [107].

Cross-talk between IGF-IR and PDGFR was described in different cellular models [108], and the dual blockade of the IGF-IR and PDGFR was suggested to be a valuable strategy for the rhabdomyosarcomas treatment [109].

Stem cell factor **(SCF)** c-kit and IGF-I/IGF-IR autocrine loops play a prominent role in the growth of small cell lung cancer. Targeting both c-kit and IGF-IR synergistically increased the antiapoptotic effect, in comparison to either of the receptor inhibition alone using a downstream pathways ERK1/2 dependent [110].

1.9 Hypothesis

Receptor cross-talk involving IGF-IR results in increased growth, survival, migration, and therapy resistance in ATRT cells

1.9.1 Overall aim

To identify a unique growth stimulatory mechanism in ATRT cells, particularly the receptor cross–talk pathways that synergize with pro-survival mechanisms of the tumor cells.

1.9.1.1 Specific aims

1. To identify the nature of novel IGF-IR cross-talk in ATRT cells.

   Identification of such a cross-talk will entail certain consequences, including:

   a. Changes in apoptotic pathways
   b. Effect on cell migration
   c. Changes in drug sensitivity by promoting resistance

2. To identify targeted agents for effective inhibition of IGF-IR cross-talk.

   This will provide appropriate strategies that may improve response to therapy.

Chapter Two: Materials and Methods

2.1 Cell lines and cell culture

BT12 and BT16 cell lines were established from infants with CNS ATRT, and were generously provided by Drs. Peter Houghton and Jaclyn Biegel (Nationwide Children's Hospital, Columbus, Ohio and The Children's Hospital of Philadelphia, respectively). These cell lines have been used extensively in preclinical studies in ATRT. These cell lines were cultured in Opti-MEM medium (Gibco, Invitrogen Corporation, Burlington, Ontario) containing 5% Fetal Bovine Serum **(FBS)**, 100 units/ml penicillin, and 100 units/ml streptomycin (Gibco).Cells were trypsinized with 0.25% Trypsin-ethylendiaminetetraacetic acid **(EDTA)** in Ca^{2+} and Mg^{2+} free balanced salt solution (Gibco) every three to five days. All cell cultures were maintained in incubators at 37^0 C in a humidified atmosphere, with 5% CO2.

2.2 Antineoplasic agents

AEW541 was kindly provided by Novartis Pharma (Basal, Switzerland). A stock solution of this agent was made in dimethyl sulfoxide **(DMSO)** (20 mM), and stored at -20^0C. This solution was subsequently diluted in culture medium for each experiment. All other targeted therapeutic agents were synthesized, checked for purity, and provided by Chemie tek (Indianapolis, USA). These agents were dissolved in DMSO to a final concentration of 10 mM, stored frozen at -20^0C, and diluted appropriately in culture medium at the time of study. Control cultures were made by diluting DMSO with culture medium identical to that of experimental groups. Table 2.1 includes the targeted chemotherapeutic agents used and their mechanism of action.

Mechanism of action	Inhibitor
IGF-IR inhibition	AEW541 BMS-745807 OSI-906
VEGFR-2 inhibition	Axitinib Foretinib Vandatinib AV951

Table 2.1 Chemotherapeutic agents used against ATRT cell lines.

2.3 Cell growth stimulation assay

ATRT cell lines were cultured at 5 X 10^3 cells/well in 96-well plates, and incubated with increasing concentrations of IGF-I (Sigma-Aldrich) ranging from 1 X 10^{-6} to 10 ng/ml. Corresponding DMSO concentrations were used as controls. After four days in culture at 37ºC in a humidified incubator containing 5% CO_2, cell survival was quantified by automated cytometer (Celigo) as described previously [111]. In the same experiment, each experimental point was repeated in duplicate or triplicate. Experiments were repeated at least twice. Cell growth results are expressed as percentage (±S.E.) of the growth of their relative controls.

2.4 Cell growth inhibition assays

ATRT cells were trypsinized and the viable cells were counted using trypan blue, and a hemocytometer. The cells were then placed in 96 well plates (Grenier Bio One, Monroe, NC) at a concentration of $5x10^3$ cells per well. Increasing concentrations of study agents were added to these wells, to a final volume of 200 ml per well. Corresponding dilutions of the vehicle DMSO were used as controls. After four days in culture, cell survival was quantified by automated cytometer (Celigo, Cyntellect Inc., San Diego, CA, USA), according to the manufacturer's protocol [111]. In the same experiment, each experimental point was repeated in duplicate or triplicate. Experiments were repeated at least twice. Cell growth results are expressed as percentage (±S.E.) of the growth of their relative controls. The half maximal inhibitory concentration **(IC_{50})** values were calculated for each agent based on individual cytotoxicity plots.

2.5 Drug combination studies

TheIC_{25} concentration of AEW541 (i.e., the amount that induced 25% cell death by itself) was added to cultures containing increasing concentrations of the second agent. The

new IC_{50} values corresponding to the combination were then calculated and used to derive combination index **(CI)** values as described by Chou and Talaly [112]. CI provides quantitative measure for the extent of drug interaction. The following formula was used to calculate the CI:

$$CI = C_{A,\,x} / IC_{x,\,A} + C_{B,\,x} / IC_{x,\,B}$$

$C_{A,\,x}$ and $C_{B,\,x}$ are the concentrations of drug A and drug B used in combination to achieve x% drug effect. $IC_{x,\,A}$ and $IC_{x,\,B}$ are the concentrations for single agents to achieve the same effect. A CI of less than 1 indicates synergy between the two agents under the experimental conditions used.

2.6 Preparation of cell lysates

ATRT cells were grown in 6 well plates (Nunc, Rochester, NY) at an initial seeding density of $2x10^5$ cells/well for 12-24h. At approximately two-third confluence the culture medium was replaced with serum free media and cells were serum starved overnight prior to each experiment. Chemotherapeutic agents, IGF-I and deferoxamine (Sigma-Aldrich) were added at the desired concentration and time, for the corresponding experiment. The cells were then washed twice with cold phosphate buffered saline **(PBS)**, pH 7.4. Lysis and protein extractions were carried out by the addition of 400 µl/well of cell lysis buffer containing 50 mM Tris, 5 mM EDTA, 0.1%SDS, 1% Triton X-100, 0.5% Sodium Deoxycholate (Sigma-Aldrich), and protease and phosphatise inhibitors (Sigma-Aldrich) at concentrations suggested by the manufacturer. Following incubation of the plates on ice for 10 min, cells were detached from the plate by gentle repeated pipetting. The cell suspensions were centrifuged in a refrigerated bench top centrifuge for 10 min at 14,000 rpm and the supernatants were collected. Protein concentrations were determined using

Pierce BCA Protein Assay Kit (Pierce, Rockford, IL) and used in subsequent antibody arrays and Western blot analysis.

2.7 RTK arrays

Nitrocellulose membranes, each containing 42 different anti-RTK antibodies and six controls printed in duplicate, were obtained from R&D systems (Minneapolis, MN). Cell lysates from untreated and IGF-I treated (50 ng/ml for 30 min) cells were incubated with these arrays and probed with anti-phospho tyrosine- horseradish peroxidise **(HRP)** detection antibodies, as described in manufacturer's instructions. Followed by Chemiluminescence substrate (Amersham, Piscataway, NJ), and developed by exposure to x-ray film (Christie InnoMed, Montreal, QC).The images from the X-ray films were captured with a digital camera and analyzed using Image J visual analysis software [113].

2.8 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western blotting

An aliquot of 25-40 μg of cellular proteins prepared as above were separated on an 8-12% SDS-PAGE acrylamide gel, and then transferred onto nitrocellulose membranes (Bio-Rad, Mississauga, ON). The membranes were blocked with 5% skim milk in PBS containing 0.1% Tween-20 (Sigma-Aldrich) for 1h at room temperature with gentle shaking. For detecting proteins expression or activation, membranes were incubated with different primary antibodies for 2h at room temperature or overnight at 4^0C (table 2.2). This was followed by washes with PBS and incubation with appropriate secondary antibodies (Sigma-Aldrich) for 2h at room temperature (table 2.3), followed by Chemiluminescence substrate (Amersham, Piscataway, NJ) and developed by exposure to x-ray film (Christie Inno Med, Montreal, QC).

2.9 Antibodies

Table 2.2 Primary antibodies

Antibody	Species	Company	Dilution
IGF-IR β	Rabbit polyclonal	Santa Cruz Biotechnology	1:1000
Phospho-IGF-IR	Rabbit polyclonal	Santa Cruz Biotechnology	1:1000
VEGFR-2	Rabbit polyclonal	Millipore	1:1000
Phospho-VEGFR-2	Rabbit polyclonal	Millipore	1:1000
ERK 1/2	Rabbit polyclonal	Cell Signalling Technology	1:2000
Phospho-ERK1/2	Mouse monoclonal	Santa Cruz Biotechnology	1:1000
VEGF	Mouse monoclonal	R&D Systems	1:1000
HIF-1α	Mouse monoclonal	Santa Cruz Biotechnology	1:1000
AKT	Rabbit polyclonal	Santa Cruz Biotechnology	1:1000
Phospho-AKT	Mouse monoclonal	Santa Cruz Biotechnology	1:1000
PARP	Rabbit polyclonal	Cell Signalling Technology	1:1000
Caspase-3	Rabbit polyclonal	Cell Signalling Technology	1:1000
Caspase-7	Rabbit polyclonal	Cell Signalling Technology	1:1000
Actin	Rabbit polyclonal	Sigma-Aldrich	1:10000

Table 2.3 Secondary antibodies

Antibody	Species	Company	Dilution
Anti-mouse IgG	Goat polyclonal	Sigma-Aldrich	1:5000
Anti-rabbit-IgG	Goat polyclonal	Sigma-Aldrich	1:5000

2.10 Vascular endothelial growth factor enzyme-linked immunosorbent assay (ELISA)

Vascular endothelial growth factor ELISAs were performed according to the manufacturer's instructions (Quantikine, human VEGF ELISA, R&D systems, MN, USA). Before the experiment was started, confluent ATRT cell cultures were held for 24 hours in serum-free medium. The medium was exchanged to fresh serum-free medium again. The cells were then stimulated with or without IGF-I (100ng/ml) for 6 and 12 h. Conditioned media of the cells were brought on a plate coated with anti-VEGF. After 2 h, the plate was washed and an antibody against VEGF conjugated to HRP was added. After another 2 h, the plate was washed and the substrate solution was added for 30 min. Then the reaction was stopped and the fluorescence was read on an ELISA reader at 450 nm with a correction set to 540 nm. The results were then plotted against the standard curve to become the actual concentrations. The amount of VEGF protein was correlated to total protein determined by the Pierce BCA Protein Assay Kit (Pierce, Rockford, IL). All experiments were carried out in triplicate. The cells were counted at the end of the experiment by automated cytometer (Celigo), as described previously.

2.11 In vitro cell migration assay ("scratch" test)

The scratch test to quantify inhibition in cell migration was performed as described previously [114]. ATRT cells were plated in six well culture plates (Nunc). Cells were grown to confluence, washed with serum-free medium, and serum starved overnight. On the day of the assay, the cells monolayer was scraped in a straight line with a 10 µl pipette tip and the culture medium was replaced with 3 ml of serum free medium. Cells were treated with Axitinib (1µM) or AEW541 (1µM) alone or in combination with IGF-I

(50ng/ml). In other sets of experiments, cells were treated with combination of Axitinib (1μM) and AEW541 (1μM) alone or in combination with IGF-I (50ng/ml). Pictures of the same scratch area were taken at various time points (at 0 h, 8 h and 24 h) using an inverted microscope; images were analyzed using Image J visual analysis software [113].

2.12 Small interfering RNA (siRNA) and transfection

Silencer validated IGF1R small siRNA was purchased from (Invitrogen, CA, USA). ATRT cells were seeded one day ahead to give optimal cell density ($2x10^5$cells/well) for high transfection efficiency. On the day of transfection, ATRT cells were washed once with PBS, and transfected at 40–50% confluency with 20nM IGF-R SiRNA or a negative control No.1 siRNA (Invitrogen, CA, USA) using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's instructions. Transfection was carried out in 6 well plates in culture medium without antibiotics. In a separate tube, 30 μl of lipofectamine was gently mixed with 1 ml of the basal medium. After 5 min incubation at room temprature, DNA and lipofectamine diluents were mixed gently and incubated at room temperature for 20 min. The mixture was then added to the cells and mixed gently by rocking the plate back and forth. Cells were incubated at 37°C in 5% CO2 and transfection efficiency was assessed after 48 h. To study activation of signalling pathways, cells were starved overnight after transfection and then stimulated with IGF-I 50ng/ml for 30 min. Subsequently, cell lysates were prepared and subjected to immunoblotting.

Chapter Three: Results

3.1 Expression and signalling of IGF-IR in ATRT cells

In order to identify the presence and activation of IGF-IR in ATRT cell lines. BT12 and BT16 cells were grown in Opti-MEM medium supplemented with 5% FBS. After reaching approximately 75% confluence, the cells were serum depleted for 24 h, and then stimulated with 50ng/ml IGF-I for different time point (5, 10,15,20,30 min). Total protein lysates were evaluated by Western blot analysis, and examined for expression and phosphorylation of IGF-IR using total and phospho-specific antibodies. Treatment with IGF-I resulted in rapid tyrosine phosphorylation of the IGF-IR β subunit for the indicated time points (figure 3.1). Phosphorylation of IGF-1R was detectable 5 min after ligand exposure. While it peaked at 10 min in BT12 and at 20 min in BT16, it was still detectable at 30 min. Expression of actin was used as the loading controls.

3.2 Growth stimulatory effect of IGF-I on ATRT cells

Insulin-like Growth Factor-I is a known mitogen whose over-expression promotes tumor growth [54]. It is suggested that IGF-I can act in an endocrine, paracrine, or autocrine fashion to regulate cell growth, survival, and differentiation. BT12 and BT16 cells were cultured at 5 X 10^3 cells/well in 96-well plates for 4 days, with increasing concentrations (0.0001–10 ng/ml) of the IGF-I in serum free medium. Cell growth was quantified by an automated cytometer (Celigo). We observed that IGF-I resulted in a significant increase in the proliferation and survival rate compared to controls in a dose dependant manner starting from 0.1 ng/ml (figure 3.2). This suggests that the growth promoting activity of IGF-I is due to an interaction with IGF-IR as shown in the previous results.

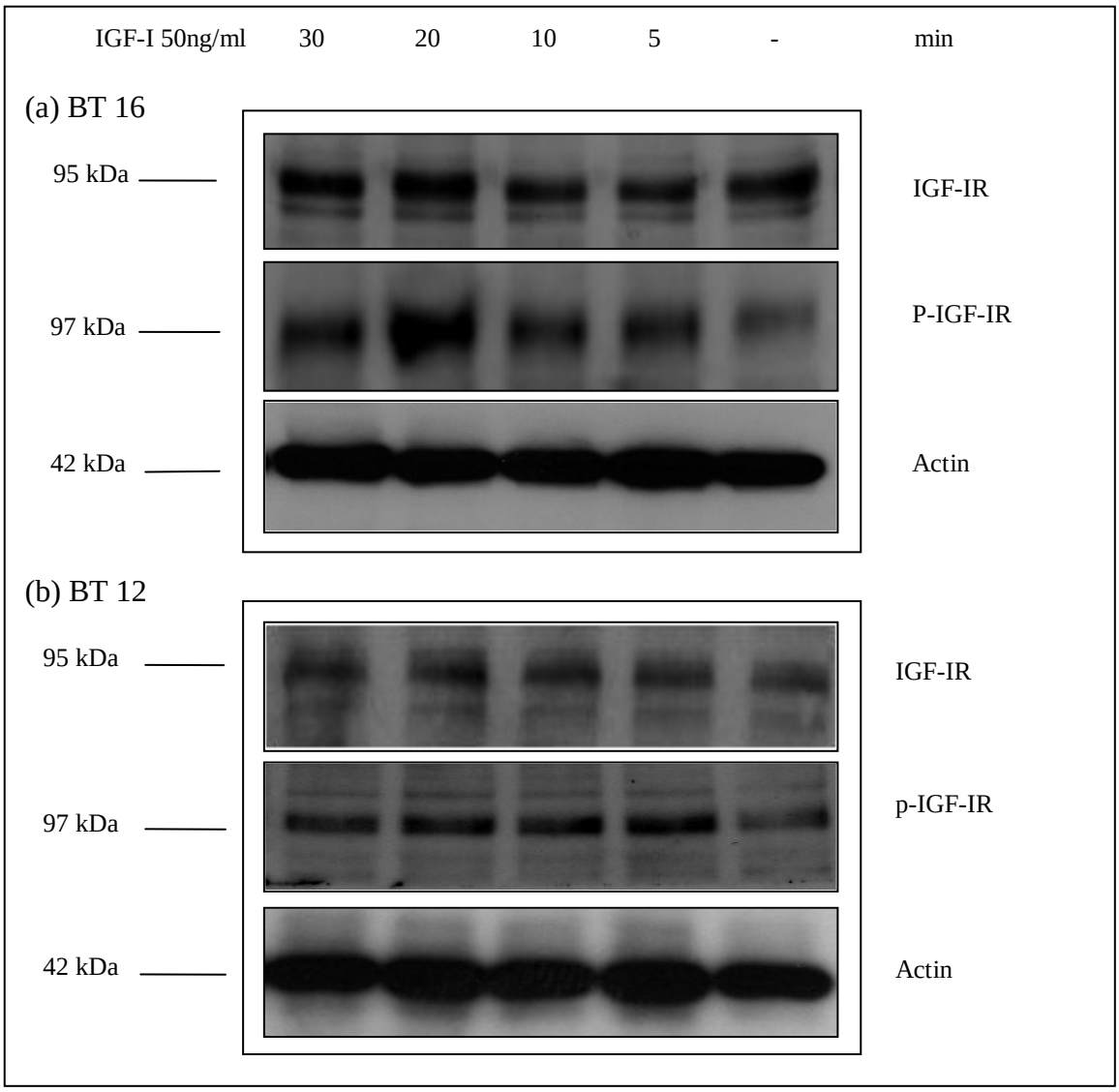


Figure 3.1 Expression of total and phosphorylated IGF-1R.

ATRT cells were treated with IGF-I (50ng/ml) for different time points (5, 10, 20, and 30min). Cell lysates (40μg) were analyzed by Western blotting for phosphorylated IGF-IR and total IGF-IR levels.

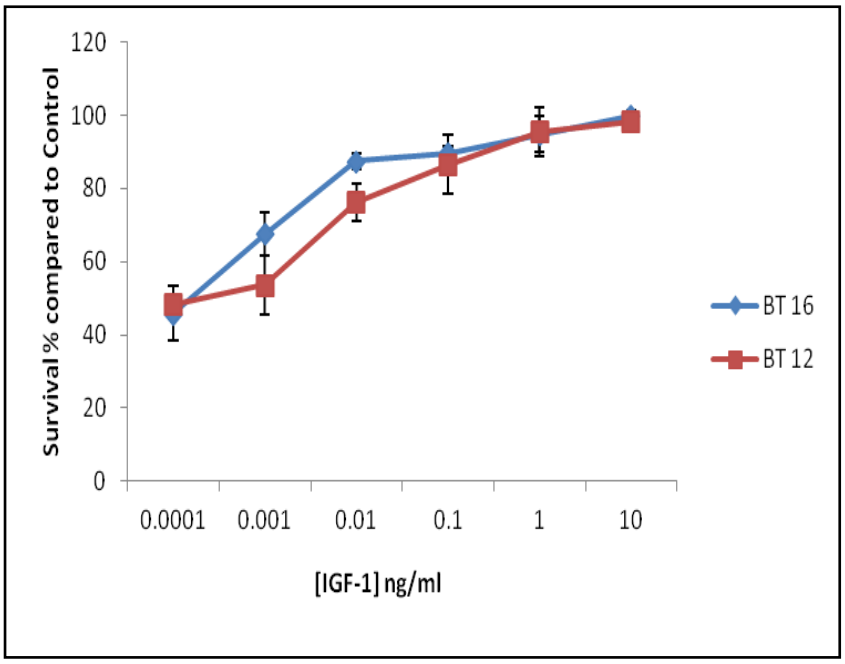


Figure 3.2 Effect of increasing IGF-I concentrations on ATRT cell survival.

The incubation of ATRT cells for 4 days with increasing concentrations of IGF-I (0.0001–10 ng/ml) resulted in a significant and dose dependent cell growth, starting from 0.1 ng/ml. Maximum stimulation was reached at 1ng/ml. Data are from three separate experiments. Increase in viable cell numbers is expressed as a percentage (±S.E.) of the untreated control.

3.3 Effect of IGF-IR inhibition on ATRT cells

Experiments were done to examine the ability of targeted small molecular weight inhibitors (AEW541, OSI906, and BMS-745807) to inhibit the growth of ATRT cells. These agents were evaluated using *in vitro* cytotoxicity assays. Agents were selected based on their known activities in other tumor cell systems, as well as their potential to be used in human clinical trials. Cells were incubated with increasing concentrations of each individual agents or DMSO as a control. After 4 days in culture, cell growth was quantified by automated cytometer (Celigo) as previously described in the materials and methods. Growth inhibition effects were plotted as as a dose-effect curve. Results presented in figure 3.3 show significant reductions in the growth of ATRT cells by each agent. Median Inhibition Concentration (IC_{50}) was calculated by plotting [the number of viable cells with treatment divided by the number of viable cells without drug treatment] x100, versus corresponding drug concentrations. Table 3.1 provides the IC_{50} values obtained in the cytotoxicity studies. Data provided in this table show drug sensitivity values across the two cell lines. It has been shown that BT16 is sensitive to BMS-745807 with IC50 of 0.5μM but required higher concentrations of the other two agents. BT12 showed sensitivity to AEW541 and BMS-745807 with IC_{50} of 0.5μM. These results suggest a critical role for IGF-IR activation in the growth and survival of these cells.

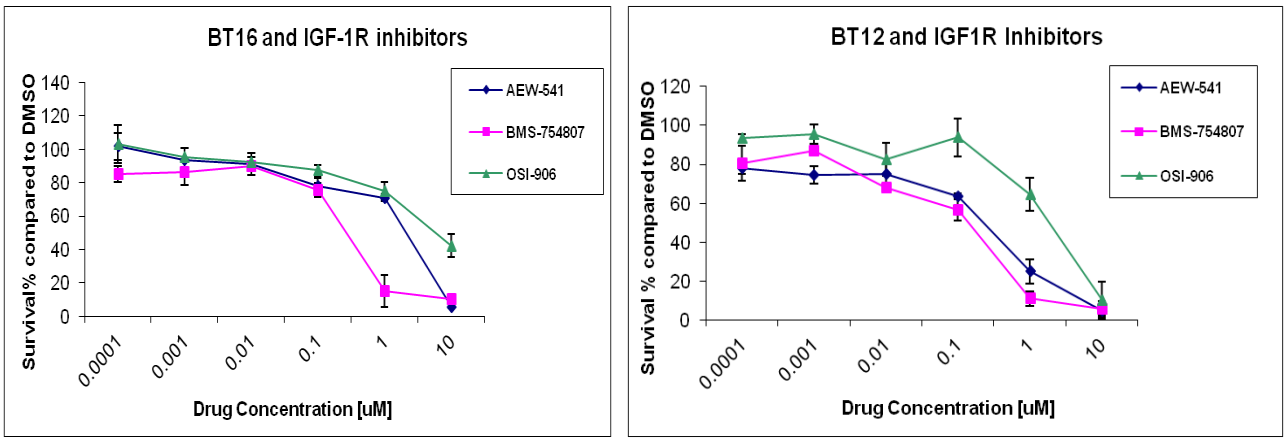


Figure 3.3 Evaluation of the ability of the IGF-IR targeting agents to inhibit the growth of ATRT cells.

Cells were grown in 96 well plates with increasing concentrations of each individual agent (AEW541, OSI906, and BMS-745807) or DMSO control were added to triplicate wells. After 4 days in culture, cell growth was quantified by automated cytometer as described above. Results presented in figure 3.3 show that IGF-IR inhibition leads to reduction in the growth of ATRT cells.

IGF-IR inhibitor	BT16 IC_{50} (µM)	BT12 IC_{50} (µM)
AEW541	5.4	0.5
BMS-745807	0.5	0.5
OSI-906	10	7.5

Table 3.1 Analysis of sensitivity of ATRT cells for various IGF-IR inhibitors.

The IC_{50} values are from a single complete study, and the overall trend is representative of three separate experiments.

3.4 Identification of potential cross-talk involving IGF-IR in ATRT cells

The next set of experiments was carried out to identify the potential cross-talk between IGF-IR and potential pathways involved in tumor growth, metastasis and angiogenesis. A screening of receptor tyrosine kinases was performed, in order to identify the presence of phosphorylated receptor tyrosine kinases using Human phospho-receptor tyrosine kinase arrays (figure3.4). This antibody array technique provides an effective tool to screen for activation of receptor tyrosine kinases (RTKs). ATRT cells were treated with IGF-I (50 ng/ml) after serum starvation overnight. Lysates from IGF-I treated and untreated cells were prepared as mentioned previously, and incubated with antibody arrays carrying capture antibodies to 42 different RTKs and six controls printed in duplicate as described in manufacturer's instructions (R&D Systems). The phosphorylation status of each RTK bound to its corresponding spot was detected by anti-phospho-Tyrosine-HRP detection antibody and chemiluminescence. This study showed increased phosphorylated IGF-IR and Insulin Receptor (IR) as expected with IGF-I stimulation but also showed the unexpected finding of VEGFR-2 activation as a result of IGF-I treatment (figure3.5 a, b).

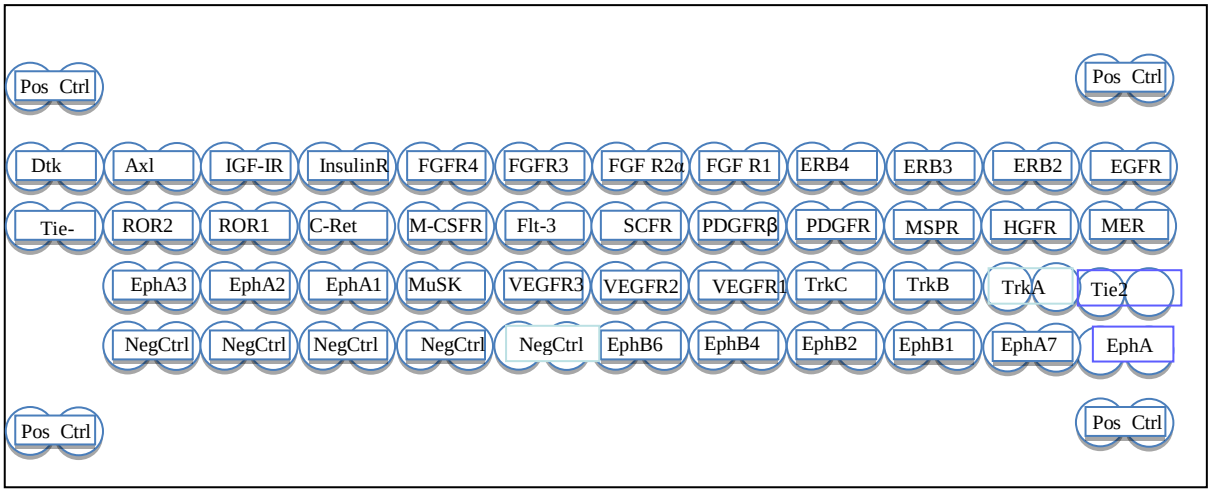


Figure 3.4 a diagrammatic representation of the array map showing the positions of capture antibodies to different RTKs.

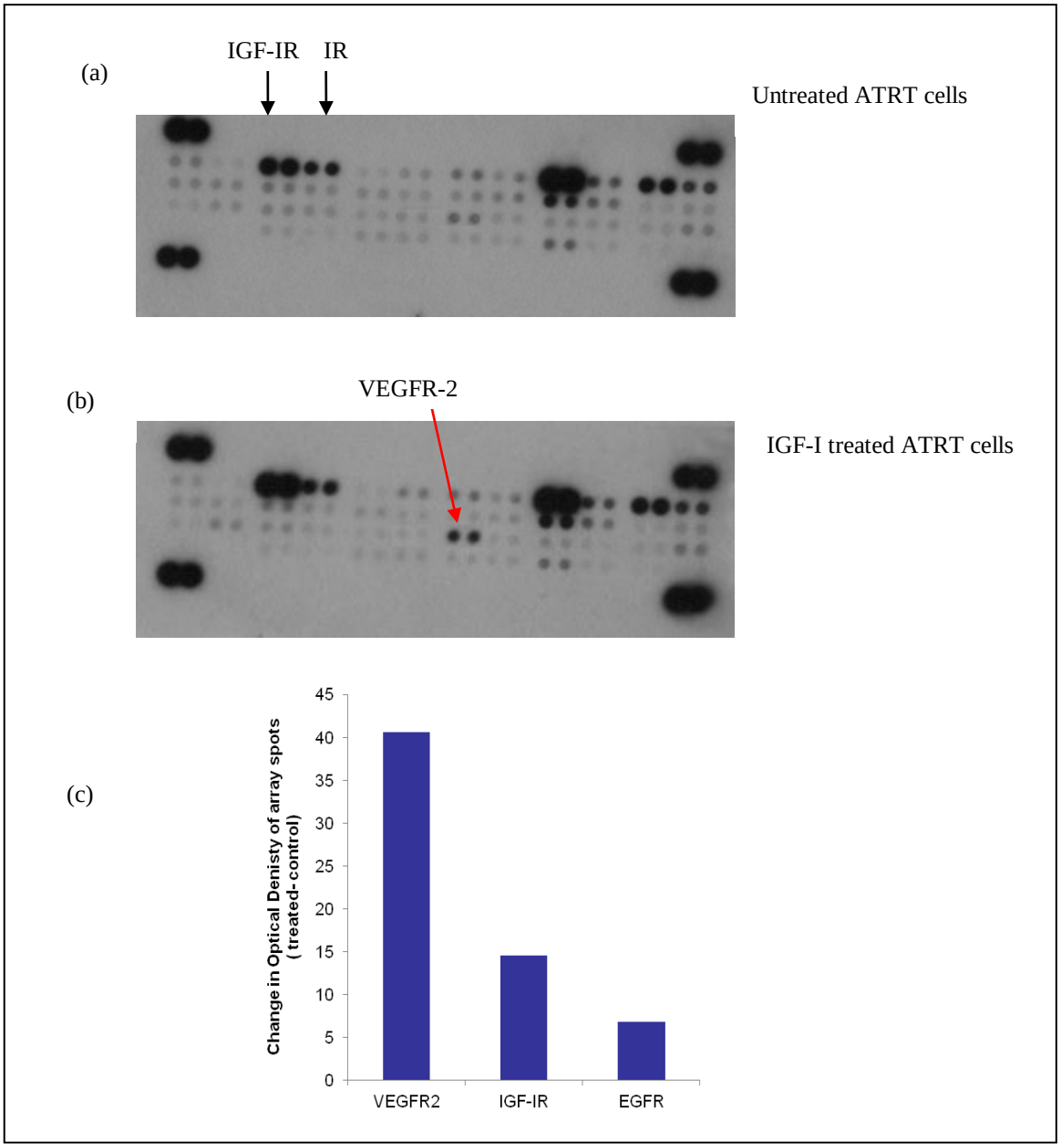


Figure 3.5 Identification of potential IGF-IR cross-talk in ATRT cells.

Cell lysates from untreated (a) and IGF-I treated (b) cells were incubated with RTK antibody arrays. Arrows point to the respective RTKs in which an increase in signal intensity, suggesting phosphorylated tyrosine, was noted. Each spot was then quantified and the relative decrease in signal was represented as a histogram (c). Data presented in these figures indicate the phosphorylation of IGF-IR, IR, EGFR and VEGFR-2.

3.4.1 Validation of RTK array findings

To validate the findings from the RTK arrays, we investigated the expression of VEGFR-2 phosphorylation status in ATRT cells in response to IGF-I treatment by Western blot analysis. BT12 and BT16 cells were serum-starved overnight and then stimulated with IGF-I (50 ng/mL) for different time points (5, 10, 20, 30 and 60 min). Lysates were obtained as previously described, and probed for the expression of total and phosphorylated forms of VEGFR-2 and IGF-IR. IGF-IR phosphorylation was induced within 5 minutes, while total IGF-IR levels were unaltered. Importantly, phosphorylation of VEGFR-2 was also induced within 5 minutes of IGF-I exposure, suggesting potential cross-signaling from IGF-IR to VEGFR-2 in both ATRT cell lines (figure 3.6).

We also examined downstream consequences of this effect by looking at the modulation of one of the critical signalling cascades, known to involve both IGF-IR and VEGFR-2 -mediated mitogenic, and antiapoptotic signalling. This includes the activation of ERK1/2. In the same experiment (figure3.6), IGF-I activated the mitogenic MAP-kinase pathway in a time dependent manner as shown by phosphorylation of ERK1/2. These results support the concept that IGF-I cross-activates VEGFR-2 signaling pathway, although the signaling molecule examined are downstream of multiple growth factor receptors. In conclusion these data suggest that IGF-I treatment in the absence of other growth factors resulted in an activation of VEGFR-2, indicating a possible IGF-IR/VEGFR-2 cross -talk in ATRT cells.

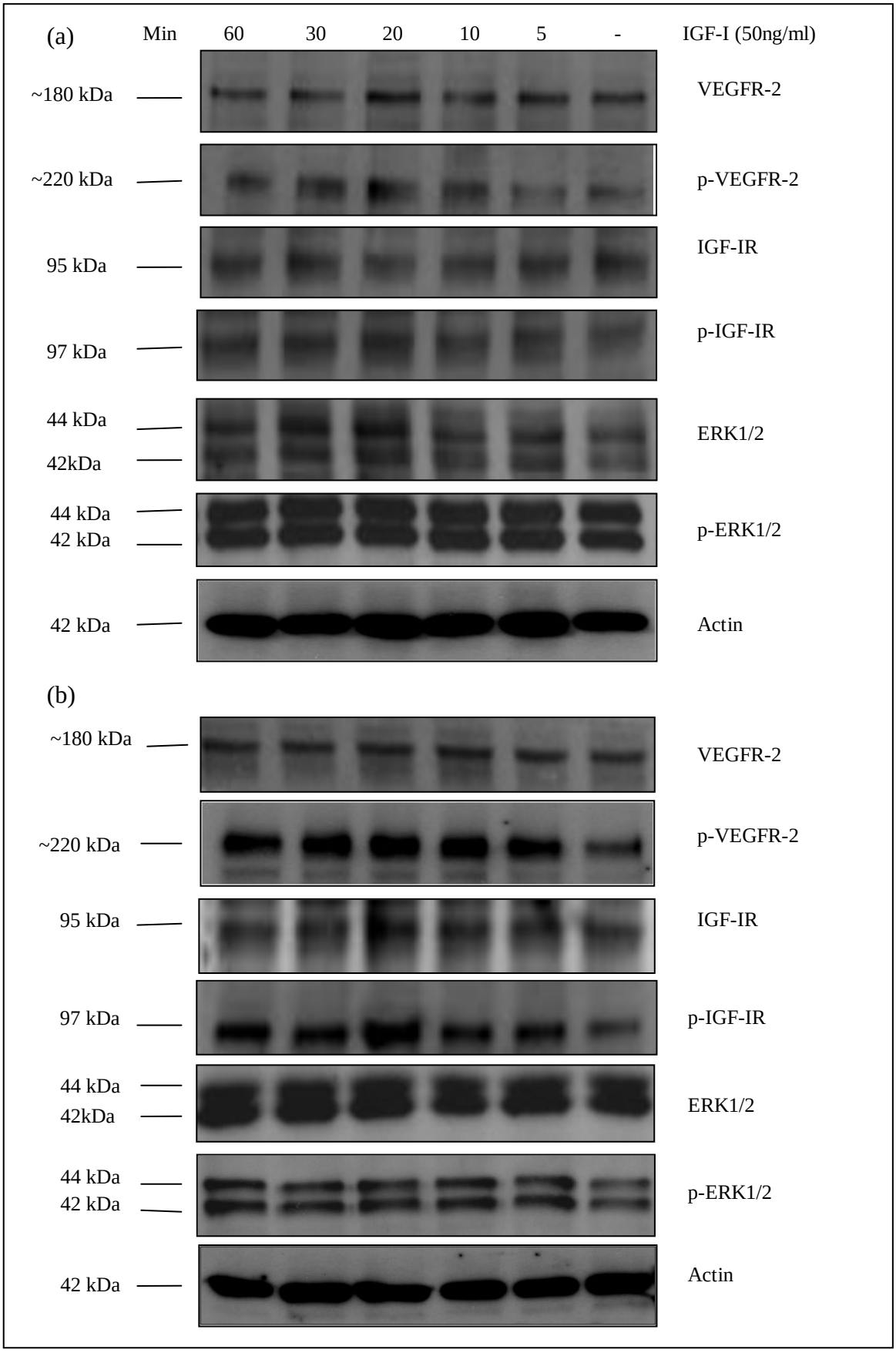

(a)
Min
60
30
20
10
5
-
IGF-I (50ng/ml)
~180 kDa
VEGFR-2
~220 kDa
p-VEGFR-2
95 kDa
IGF-IR
97 kDa
p-IGF-IR
44 kDa
42kDa
ERK1/2
44 kDa
42 kDa
p-ERK1/2
42 kDa
Actin
(b)
~180 kDa
VEGFR-2
~220 kDa
p-VEGFR-2
95 kDa
IGF-IR
97 kDa
p-IGF-IR
44 kDa
42kDa
ERK1/2
44 kDa
42 kDa
p-ERK1/2
42 kDa
Actin

Figure 3.6 Evidence of cross-talk from IGF-IR to VEGFR-2.

IGF-I induces phosphorylation of VEGFR-2. BT12 (a) and BT16 (b) cells were serum-starved overnight and then stimulated with IGF-I (50 ng/mL) for 5, 10, 20, 30, and 60 min. Cell lysates (30 μg) were immunoblotted for total and phosphorylated IGF-IR, VEGFR-2, and ERK1/2. Actin was used as loading control. IGF-I stimulated the phosphorylation of IGF-IR within 5 min in both cell lines. Importantly, phosphorylation of VEGFR-2 was also induced within 5 min of IGF-I exposure.

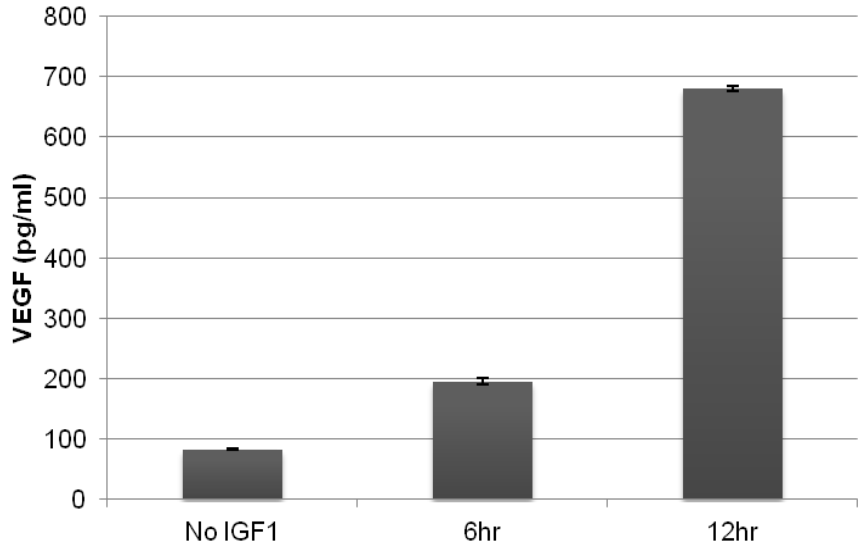
3.5 IGF-I regulates VEGF secretion in ATRT cells

It is well known that growth factors binding to their receptors tyrosine kinases may induce HIF-1α synthesis and VEGF secretion via the activation of PI3Kand MAPK pathways promoting tumor progression and metastasis [115]. HIF-1α is a transcriptional factor in mammalian cells that is selectively stabilized and activated under hypoxic conditions; it coordinates adaptive responses to hypoxia required for DNA binding and transactivation of target genes [115]. HIF-1α is primarily regulated at the level of protein stability under normoxic conditions, and is rapidly degraded by the ubiquitin–proteasome system. Furthermore, while HIF-1α degradation is inhibited under hypoxic conditions, it is also regulated by hypoxia-independent mechanisms. For example, HIF-1α has been shown to be activated in response to IGF-I in cancer cells and epithelial cell lines, thus leading to the expression of VEGF [116]. However, the role of IGF-I-induced HIF-1α expression in ATRT has not been investigated. The purpose of the following experiments is to elucidate this intracellular signaling in ATRT cells.

3.5.1 VEGF secretion in ATRT cells

The aim of this study is to evaluate IGF-I- induced VEGF secretion in ATRT cells. To test this, VEGF secretion by ATRT cells was measured by ELISA. Serum-starved ATRT cells were stimulated with or without IGF-I for 6 and 12 h. The medium was then harvested and tested for the presence of secretory VEGF. As shown in figure 3.7 a, VEGF secretion was increased by 50% to 85% by IGF-I. Quantification of cells at the end of each time point did not show significant changes indicating that proliferation is not responsible for the increase in VEGF concentration (figure 3.7 b).

(a)


800
700
600
500
400
300
200
100
0
VEGF (pg/ml)
No IGF1
6hr
12hr

(b)

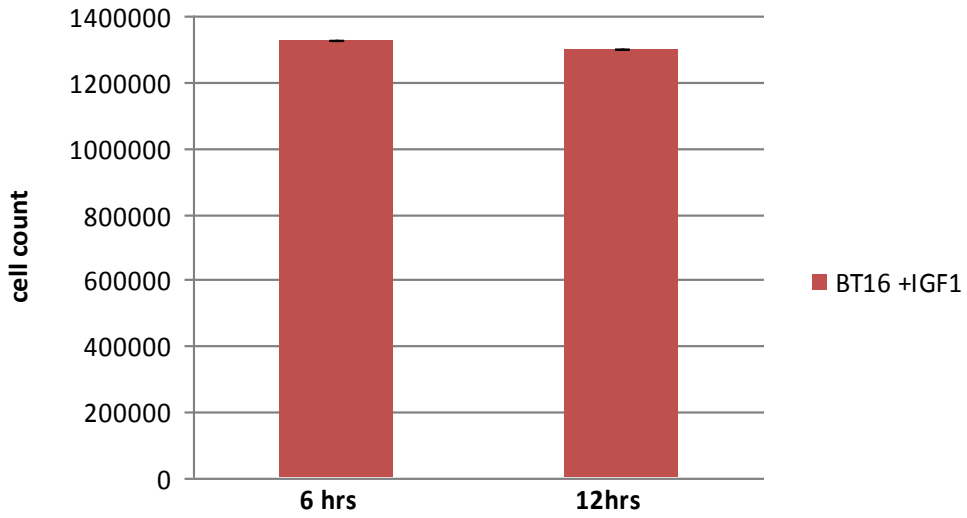

1400000
1200000
1000000
800000
600000
400000
200000
0
cell count
6 hrs
12hrs
BT16 +IGF1

Figure 3.7 IGF-1 induced secretion of VEGF by ATRT cells.

(a) Serum-depleted ATRT cells were treated with IGF-I (100 ng/mL) for 6 and 12h, the media were collected for ELISA of VEGF. The concentration of VEGF was measured as picograms per millilitre (pg/ml) and normalized to protein content of the cells. (b) At the end of each time point cells were counted using automated cytometer. Untreated cell were used as control. Each experiment was performed three times and the mean ± SD of results of triplicates are shown as error bars.

3.5.2 IGF-I stimulates the expression of HIF-1α and VEGF in ATRT cells

The following experiments were done to investigate whether the activation of IGF-I regulated HIF-1α protein expression in ATRT cells. We used the iron chelator Deferoxamine **(DFO)** (100 μM) as a positive control, because it stabilizes HIF-1α expression through inhibition of asparaginyl hydroxylase, causing increased expression of HIF-1 target genes [117]. As expected, DFO stimulated expression of HIF-1α. Exposure of ATRT-starved cells to IGF-I (100ng/ml) for 6, 12, and 24 h resulted in a time-dependent increase in levels of HIF-1α protein, with maximal effect at 6h, based on Western blotting analysis. In the same experiment VEGF protein expression was also examined. On treatment with IGF-I, VEGF levels increased, with maximal effect at 12h (Fig. 3.8 In these studies, we showed that IGF-I increases the expression of HIF-1α leading to increased VEGF secretion, which in turn may stimulate vascularization and angiogenesis through binding to its receptor VEGFR-2. These observations suggest a possible mechanism for IGF-IR/VEGFR-2 cross-talk through IGF-I.

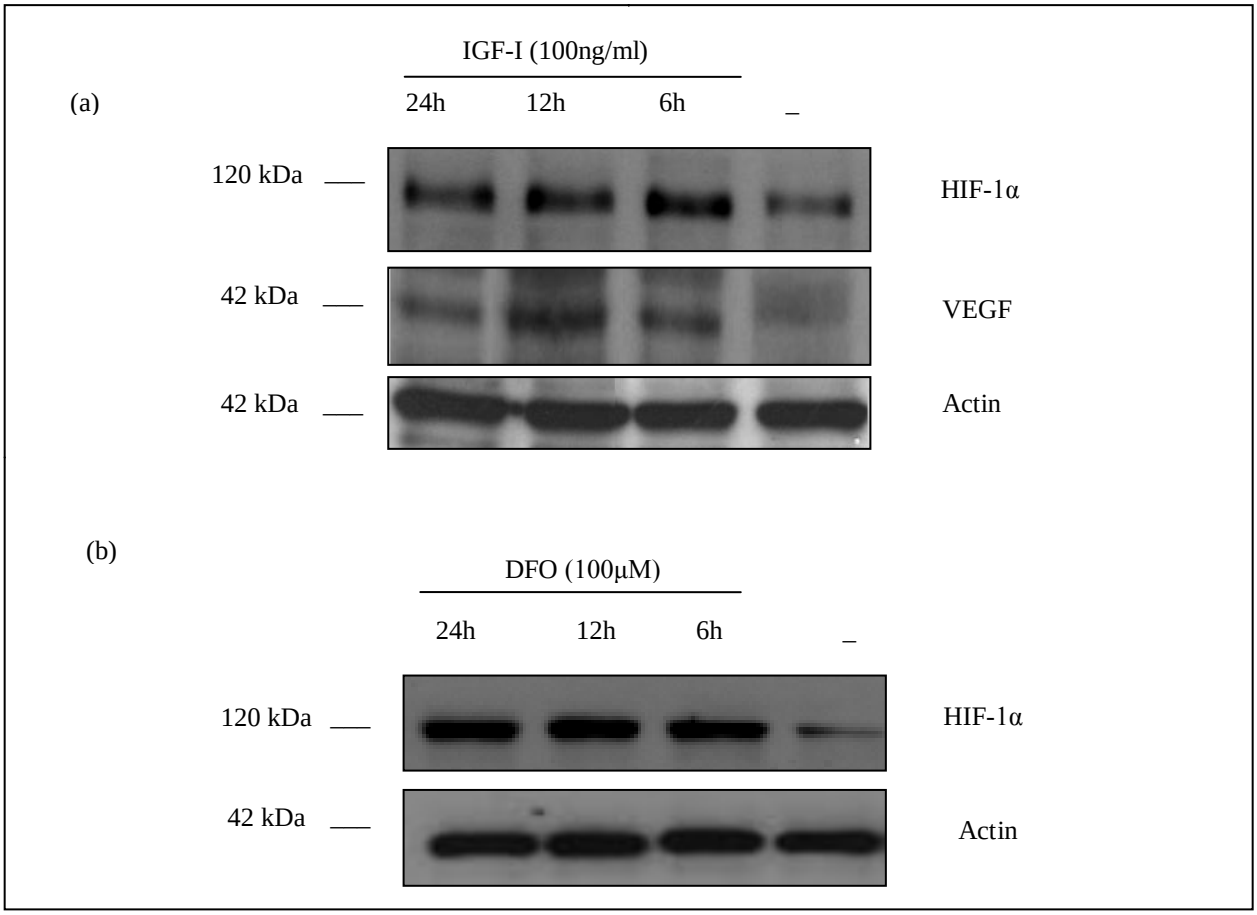


Figure 3.8 IGF-1 stimulates HIF-1α and VEGF protein expression in ATRT cells.

Representative Immunoblots showing HIF-1α and VEGF expressions after (a) IGF-I (100ng/ml) treatment and (b) DFO (100 μM) treatment for 6, 12, and 24 h.

3.6 Role of IGF-IR/VEGFR-2 cross-talk in the development of treatment resistance in ATRT cells

The following experiments were done to investigate the effects of potential cross talk involving IGF-IR and VEGFR-2. To this end we first tested whether AEW541 can block IGF-IR activation. The cells grown in growth media were transferred to serum-free media over night before adding the drug AEW541. Figure 3.9 (a) and (b) shows that 1 to 10 μM of AEW541 suppressed the levels of phosphorylated IGF-IR, and phosphorylated ERK1/2. In the second part of this study, ATRT cells were grown as above, and treated with AEW541 in the presence of IGF-I (50ng/ml) for 4h. Lysates from these cells were probed by immunoblotting. Figure3.9 (c) and (d) shows that the presence of IGF-I induced phosphorylation of VEGFR-2. ERK1/2 is located in the nodal points of growth factor–mediated cell signalling. Hence, we probed for ERK1/2, to see if its dephosphorylation status induced by AEW-541 alone will be affected by the presence of IGF-I. Addition of IGF-I stimulated ERK1/2, suggesting that IGF-I enhanced the ERK activation through VEGFR-2 signalling pathway promoting resistance to AEW541.

These data so far indicate that ATRT cells have the VEGFR-2 activation as an additional input into PI3K signaling pathway and this modulates the action of IGF-IR blocking agents, suggesting a potential mechanism involved in treatment resistance of ATRT.

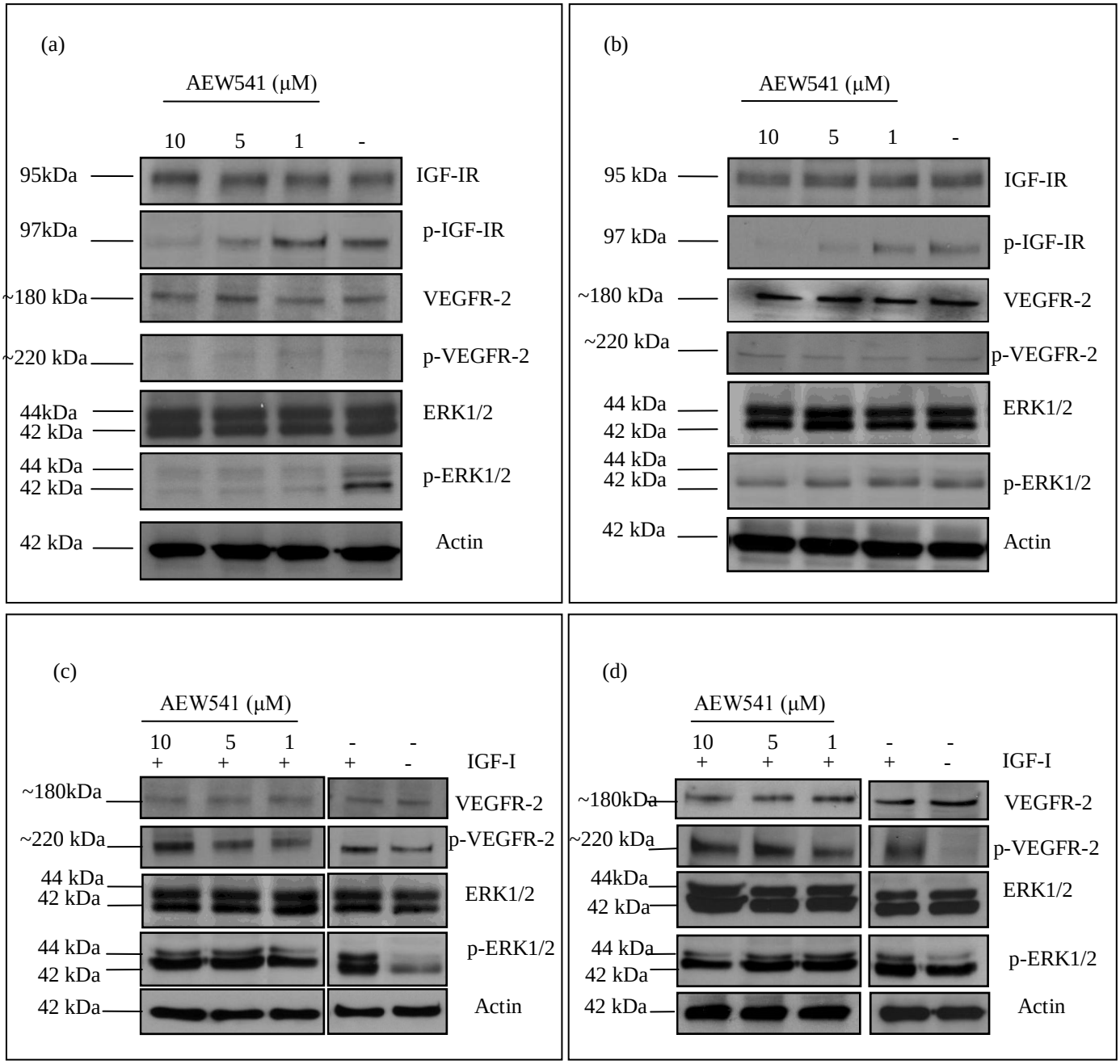


Figure 3.9 IGF-I activate VEGFR-2 and promote AEW541 resistance in ATRT cells.

(a) BT12 and (b) BT16 cells were exposed to AEW541 (1, 5, and 10 μM) as indicated for 4 hours. Cells were lysed and probed with indicated antibodies. (c) BT12 and (d) BT16 cells were subjected to increasing doses of AEW541in the presence of 50 ng/ml IGF-I. Western blotting on actin is included as a loading control.

3.7 IGF-I overcomes the effect of IGF-IR inhibitor on ATRT cells migration

Cancer progression is a multistep process that involves invasion of basement membrane by tumor cells and migration to points far from a given primary tumor mass leading to metastasis. Increasing evidence indicates that IGF-I signalling is also pivotal to cell motility and migration [118].

To confirm the effects of IGF-I on ATRT cell migration, we did a scratch wound assay. Confluent ATRT cells were serum starved overnight, wounded with a pipette tip, and then the disrupted cell layers were incubated in the presence of 100ng/ml IGF-I. In this scratch assay, the movement of cells across a scratch line is evaluated as an indication of the capability of an agent to stimulate or inhibit cell migration. Cell migration into the detection zone was quantified by counting cell number using ImageJ software (http://rsb.info.nih.gov/ij/) (version 1.4.3.67). The serum free media-treated cells showed delay wound closure. By 24 hours, IGF-I treatment led to migration of ATRT cells to almost complete closure of the wound (figure 3.10 a). This confirms that IGF-I signalling contributes to ATRT cell migration. As shown previously, IGF-I was able to activate VEGFR-2, which is known to transduce full range of responses including regulating survival, proliferation, and migration processes. Therefore, we hypothesized that IGF-I can mediate cell migration through VEGFR-2 activation. To test this, we first investigated the effect of IGF-IR inhibition on cell migration by the scratch assay, as previously described. When serum starved confluent ATRT cells were treated with AEW541 (5μM) for 8 and 24 hr, there was no significant cell migration observed in the wound area. However, the addition of IGF-I restored wound healing to levels comparable to cells treated only with IGF-I (figure 3.10 a). This suggested the possibility that IGF-I is utilizing an alternative

pathway, possible involving VEGFR-2 to stimulate cell migration. To further test this possibility, we looked at the effect of co-targeting IGF-IR and VEGFR-2 pathways on IGF-I mediated cell migration. In the scratch assay, after the serum starved confluent ATRT cells were wounded, AEW541 (5μM) and Axitinib (5μM) were added to the cells in presence or absence of IGF-I (100ng/ml), and cells migration were evaluated at 8 and 12 hr. When treated with both inhibitors, dependent loss of cell migration over the scratch line, demonstrated their combined ability to prevent ATRT cell migration. A similar trend was observed in the presence of IGF-I (figure 3.10 a). To differentiate cell proliferation versus cell migration, and thus to account for the additional cells in the scratch zone, viable cell count was measured by Alamar blue assay and a graphic representation for cell migration, and corresponding cell counts are given in (figure 3.10 b). Taken together, the results presented here provide evidence for IGF-IR /VEGFR-2 signaling cross-talk, as well as suggesting that IGF-I is a critical component of this cross-talk, with a key role in stimulating cell migration.

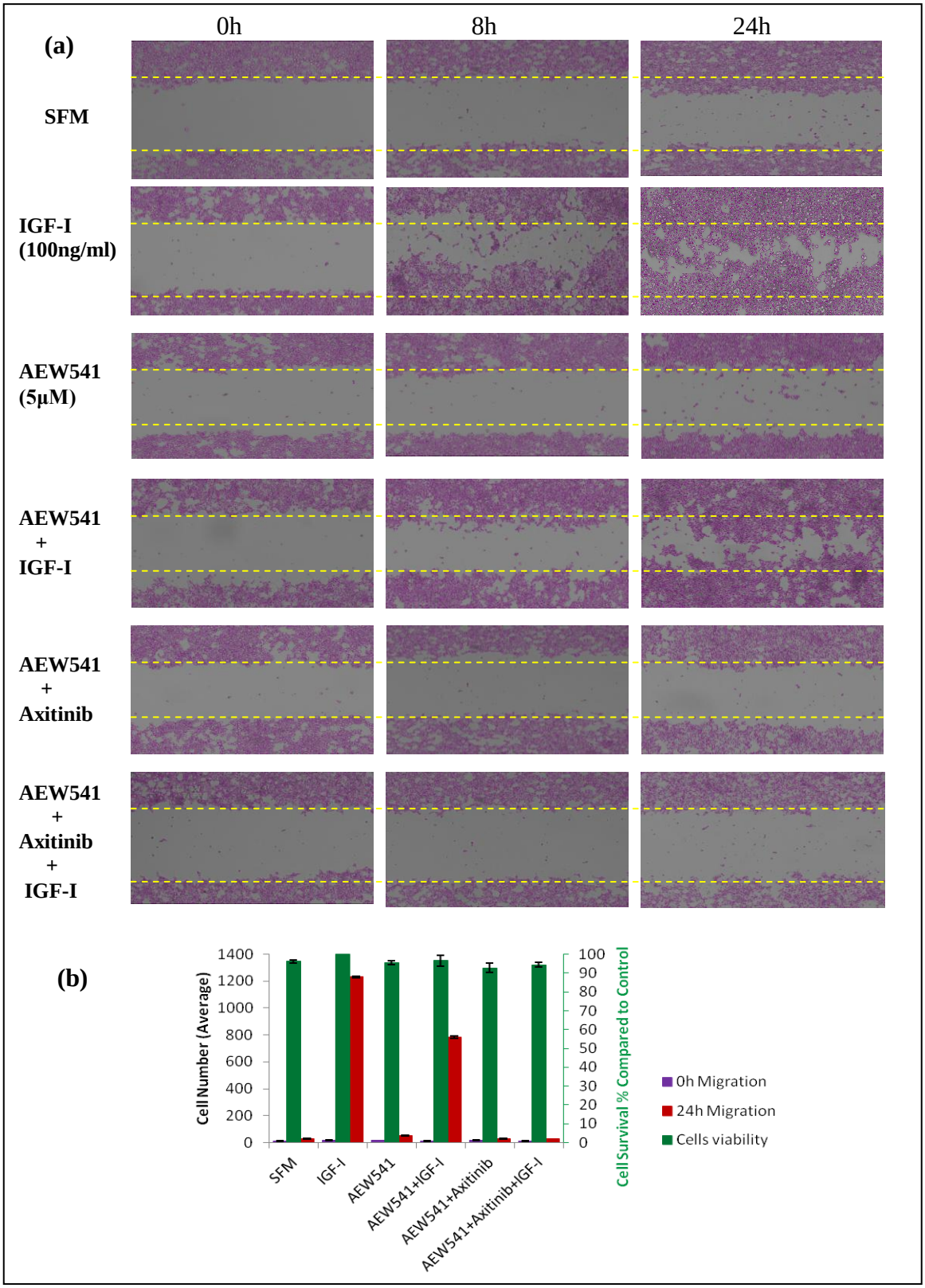

(a)
0h
8h
24h
SFM
IGF-I (100ng/ml)
AEW541 (5μM)
AEW541 + IGF-I
AEW541 + Axitinib
AEW541 + Axitinib + IGF-I
(b)
Cell Number (Average)
Cell Survival % Compared to Control
0h Migration
24h Migration
Cells viability
SFM
IGF-I
AEW541
AEW541+IGF-I
AEW541+Axitinib
AEW541+Axitinib+IGF-I

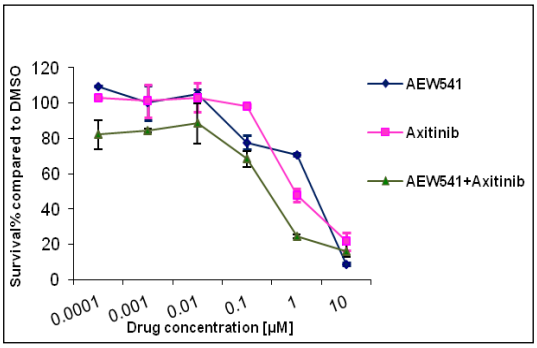
Figure 3.10 In vitro cell migration assay.

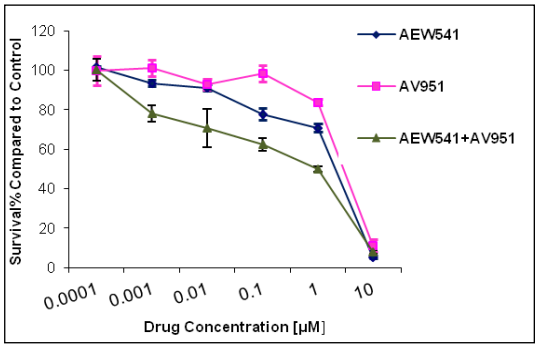
(a) ATRT cells were plated in 6-well plate and a scratch was introduced when cells were 80% confluent. Images were acquired at 0 h, 8 h and 24 h, following the in vitro scratch assay. The dotted lines define the areas lacking cells. (b)The rate of migration was measured by quantifying the total distance that cells moved from the edge of the scratch toward the center of the scratch (marked by imaginary dotted lines). After 24 h in culture, the quantity of viable cells in each condition was measured by Alamar blue assay. Data presented above is representative of three separate experiments.

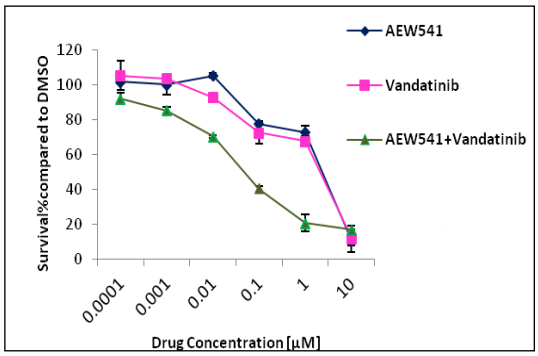
3.8 Synergetic activity of AEW541 with VEGFR-2 inhibitors

Previous studies have demonstrated that IGF-IR activity contributes to the growth and survival of ATRT cells. The antibody array analysis presented in figure 3.5 also showed the activated status of IGF-IR in ATRT cells. These data provided a mechanistic rationale to investigate the hypothesis that a combined inhibition of both IGF-IR and VEGFR-2 would show synergy against these cells. In the next set of experiments, we investigated targeted VEGFR-2 therapeutic agents and an IGF-IR inhibitor (AEW541) (in drug combination studies. Studies of AEW541 in combination with the targeted VEGFR-2 inhibitors were carried out as described in Materials and Methods. A graphic representation of cell survival when treated with drug combination is given in Figure 6. The combination indices (CI) [112], calculated from these experiments, are given in Table 2. In this analysis, a CI value equals to 1, less than 1 and more than 1 indicates additive, synergistic and antagonistic effects, respectively, between the two agents. Values presented in Table 2 show synergy between AEW541 and Axitinb, AV951 and Vandatinib in BT16 and synergy between AEW541 and Foretinib in BT12 indicate that these cell lines are most susceptible to the combined effect of IGF-IR and VEGFR-2 inhibition.

(a)


Survival% compared to DMSO
120
100
80
60
40
20
0
0.0001
0.001
0.01
0.1
1
10
Drug concentration [µM]
AEW541
Axitinib
AEW541+Axitinib


Survival% Compared to Control
120
100
80
60
40
20
0
0.0001
0.001
0.01
0.1
1
10
Drug Concentration [µM]
AEW541
AV951
AEW541+AV951


Survival%compared to DMSO
120
100
80
60
40
20
0
0.0001
0.001
0.01
0.1
1
10
Drug Concentration [µM]
AEW541
Vandatinib
AEW541+Vandatinib

(b)

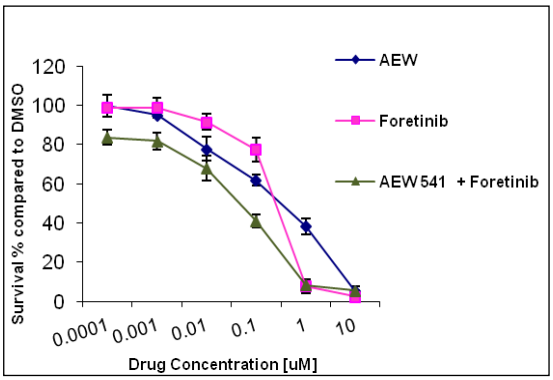

Survival % compared to DMSO
120
100
80
60
40
20
0
0.0001
0.001
0.01
0.1
1
10
Drug Concentration [uM]
AEW
Foretinib
AEW 541 + Foretinib

Figure 3.11 Drug combination study of AEW541 with VEGFR-2 inhibitors in ATRT cell lines.

BT16 (a) and BT12 (b) cells were incubated with increasing concentrations of AEW541 and a VEGFR-2 inhibitor alone or increasing concentrations of VEGFR-2 inhibitor with a constant IC_{25} concentration of AEW541. Cell growth inhibition was measured after four days in culture as describe above. The IC_{25} values of AEW541 used were 0.001 mM and 0.01 mM for BT12 and BT16 respectively that were calculated from figure3.3.

BT16		BT12	
Drug Combination	IC_{50}	Drug Combination	IC_{50}
AEW541 + Axitinib	0.6	AEW541 + Foretinib	0.1
AEW541 + AV951	0.17		
AEW541+ Vandatinib	0.18		

Table 3.2 Synergetic Activity of combined VEGFR-2 inhibition and AEW541 against ATRT cells.

IC_{50} values for single agent AEW541 and in combination with VEGFR-2 inhibitors were calculated from data presented in figure 3.11 and used to calculate combination indices according to the method of Chou and Talalay. A CI value less than 1 indicates drug synergy under the specific experimental conditions used.

3.9 Combination of IGF-IR and VEGFR-2 inhibitors significantly promotes ATRT cells apoptosis

To elucidate the mechanisms of IGF-IR and VEGFR-2 inhibition -mediated anti-proliferation/anti-survival effects, we tested whether AEW541 and/or VEGFR-2 inhibitors (Axitinib, Vandatinib and Foretinib) may induce apoptosis in ATRT cells. BT16 cells were treated with AEW541 (5μM) alone or in combination with Vandatinib (5μM)/Axitinib (5μM) for 36h, in case of BT12 cells were treated with AEW541 (5μM) alone or in combination with Foretinib (5μM) for 36h. Cell lysates were obtained and subjected to Western blot analysis. Data obtained in these studies showed that the combination of AEW541 and VEGFR-2 inhibitors enhanced PARP cleavage, and activation of caspase-7 and -3; as evidenced by the increases of cleaved caspase-7 and -3 in both ATRT cell lines (figure 3.12). These findings indicate that the detectable apoptotic effects can be used as effective biological correlates for the growth inhibition observed in response to AEW541 and VEGFR-2 inhibitors drug combination.

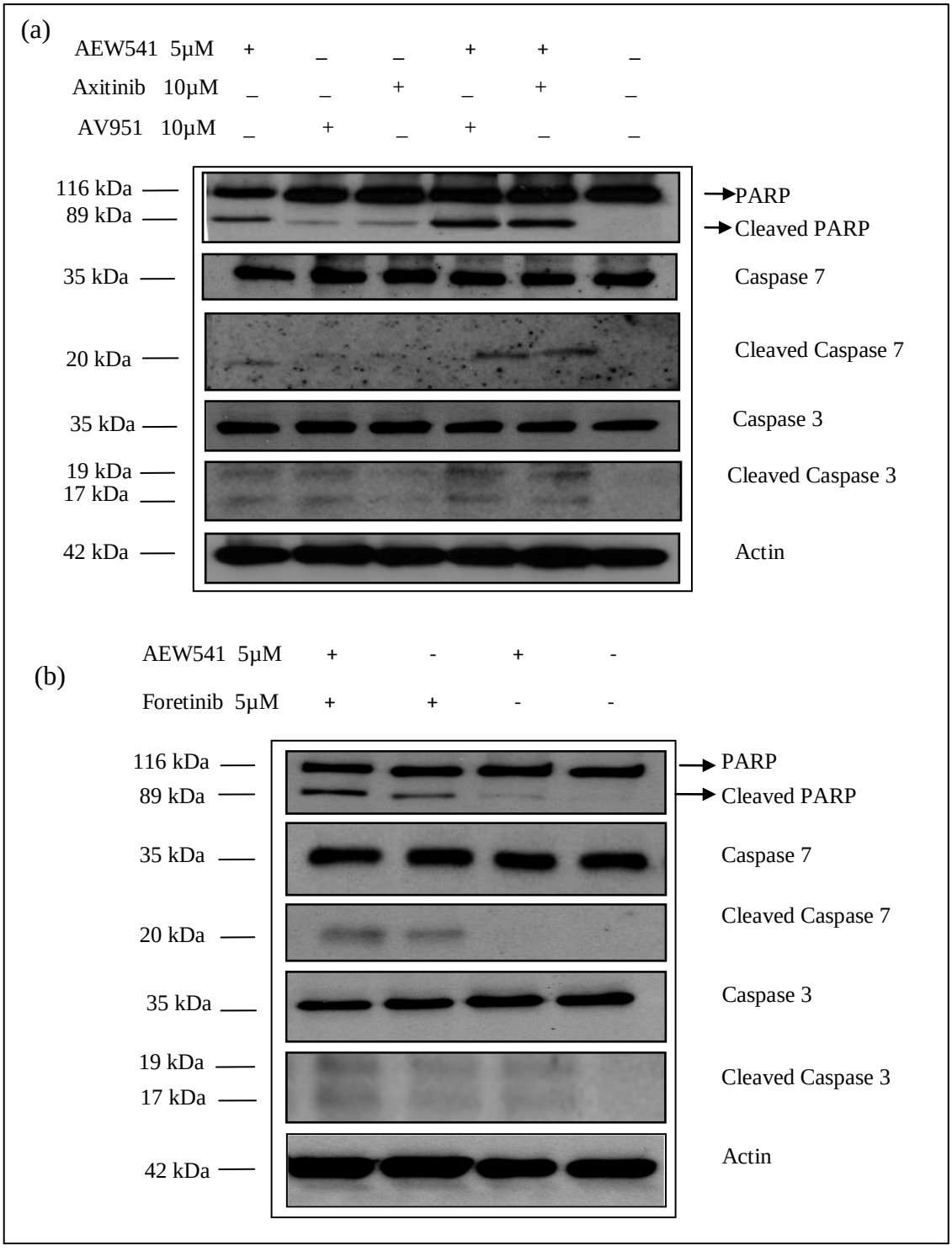

(a)
AEW541 5μM + _ _ + + _
Axitinib 10μM _ _ + _ + _
AV951 10μM _ + _ + _ _
116 kDa
89 kDa
35 kDa
20 kDa
35 kDa
19 kDa
17 kDa
42 kDa
PARP
Cleaved PARP
Caspase 7
Cleaved Caspase 7
Caspase 3
Cleaved Caspase 3
Actin
(b)
AEW541 5μM + - + -
Foretinib 5μM + + - -
116 kDa
89 kDa
35 kDa
20 kDa
35 kDa
19 kDa
17 kDa
42 kDa
PARP
Cleaved PARP
Caspase 7
Cleaved Caspase 7
Caspase 3
Cleaved Caspase 3
Actin

Figure 3.12 Effect of AEW541 and VEGFR-2 inhibitors (Axitinib, AV951 and Foretinib) combination treatment on the expression of proteins involved in apoptosis and caspase activation.

Western blot analysis was used to display changes in apoptosis-related proteins (PARP, caspase-7, and caspase-3) in ATRTcells after treatment with either AEW541 or a VEGFR-2 inhibitor (Axitinib or AV951 or Foretinib) alone, or the combinations of AEW541 and a VEGFR-2 inhibitor for 36 h.

3.10 Effect of IGF-IR knockdown on IGF-IR/VEGFR-2 interaction

This study was designed to investigate whether the VEGFR-2 axis is capable of compensating for IGF-IR loss in ATRT cells. The effect of *IGF-IR* gene silencing on VEGFR-2 expression and activity was assessed. ATRT cells were transfected with 20nM of IGF-IR siRNA, or control siRNAs for 48 h, serum-starved overnight and treated with 50 ng/ml IGF-I for 30 minutes as described in methods. Western blots of these lystaes demonstrate that siRNA successfully blocked the expression of the IGF-IR. Predictably, IGF-IR knockdown abolished IGF-I induced IGF-IR phosphorylation. IGF-IR knockdown cells treated with IGF-I caused an increase in tyrosine phosphorylation of VEGFR-2, without influencing the levels of total VEGFR-2 (figure3.13).The effect of IGF-IR knockdown on ERK activation was also investigated. Our findings show that the phosphorylation of VEGFR-2 and ERK were enhanced, and not inhibited in response to IGF-IR knockdown in presence of IGF-I as compared to IGF-IR knockdown alone. These results support a cross-talk pathway between two key receptors. The molecular basis for this interaction is unclear, but these data could indicate that this crosstalk might be associated with direct IGF-IR/VEGFR-2 interaction with IGF-I, and that VEGFR-2 compensation could mediate resistance to IGF-IR inhibition.

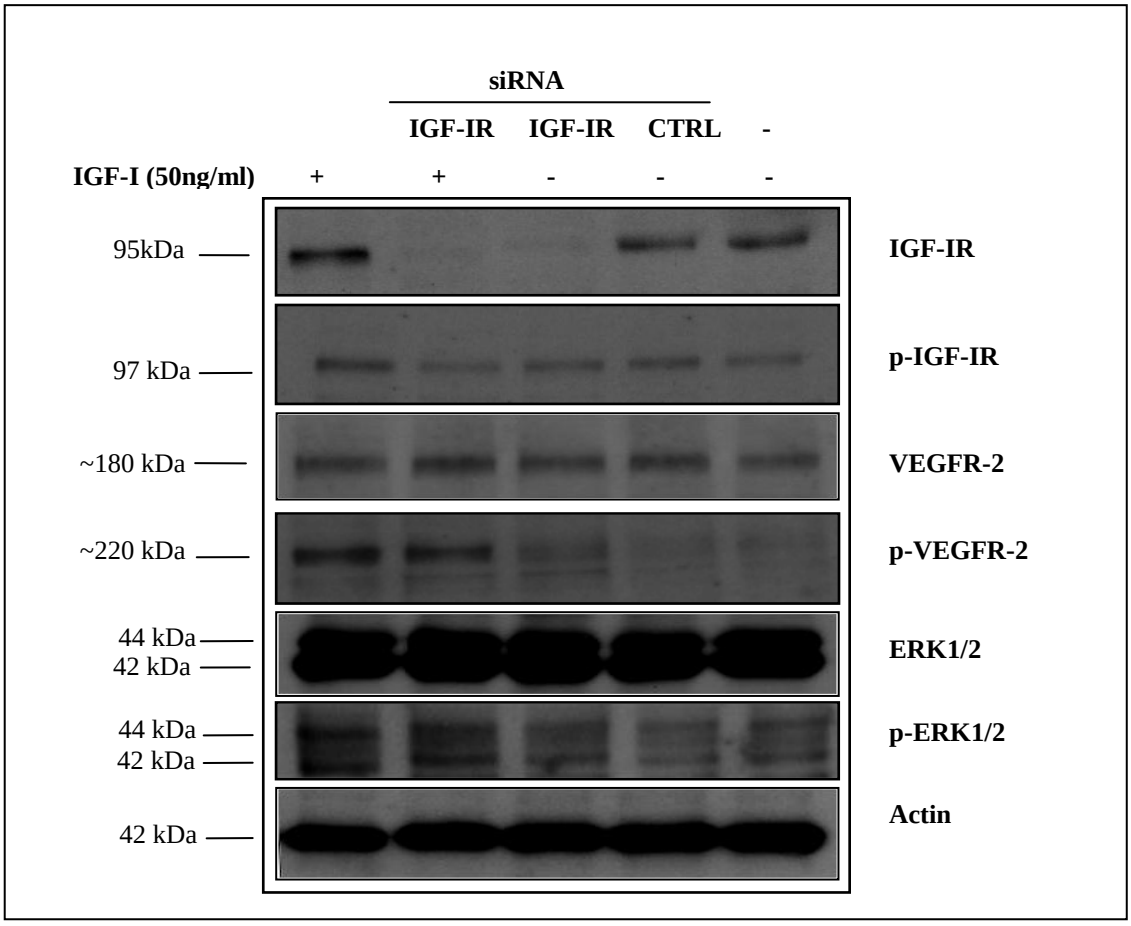


Figure 3.13 IGF-IR knockdown leads to increased VEGFR-2 activity.

ATRT cells were transfected with 20 nM IGF1R or control siRNAs. Forty-eight hours after transfection cells were serum-starved overnight and stimulated with 50 ng/ml IGF-I (+) or without IGF-1(-) for 30 min. Cells were lysed and analysed by Western blotting for levels of IGF-IR, p-IGF-IR, VEGFR-2, p-VEGFR-2, ERK1/2 and p-ERK1/2.

Chapter four: Discussion

ATRT is currently considered to be among the most difficult to cure tumors in the pediatric population. Although defects in the chromatin remodelling apparatus by the SWI/SNF complex is likely to be the key molecular feature in ATRT, the pathways and nodes that constitute deregulated growth regulatory mechanisms are critical for the identification of effective targets for future therapeutics. Although the presence of this mutation is rather undisputable, additional molecular pathways underlying ATRT development are poorly understood. The expression of IGF-I and IGF-I receptor in various pediatric brain tumors has been evaluated by Ogino and co-workers [119]. IGF-IR signalling pathway has been implicated in the development and progression of ATRT. In addition, an autocrine signalling component is present, as ATRT cells themselves produce and secrete IGF-I and express cell surface IGF-IR. Autophosphorylated IGF-IR was detected in ATRT lines, thus supporting the hypothesis that autocrine/paracrine stimulation of cell growth by IGF-IR may be involved in ATRT pathogenesis. Studies confirming activation of IGF-IR receptor mediated signalling has been shown to result in neoplastic transformation, tumor growth and survival, angiogenesis and metastasis. It is conceivable that highly expressed activated IGF-IR in CNS ATRT is involved in resistance to apoptosis. Cross-talk between different growth factor receptor families is frequently observed in tumors. This mechanism allows cancer cells to enhance downstream signalling, resulting in greatly increased proliferation, mitogenesis, and cell survival. IGF-IR has been shown to interact and cross-talk with multiple receptors, including EGFR, HER2, platelet-derived growth factor receptor, and the estrogen receptor. IGF has been linked to angiogenesis, which is essential for tumor metastasis and nutrient recruitment. In this study, we sought to identify weather novel molecular interactions occur between IGF-IR and other

receptors is present, and if it could promote ATRT aggressive behaviour. We showed the following novel findings:

(a) IGF-I stimulation induces phosphorylation and activation of both IGF-IR and VEGFR-2:

To identify the activated RTKs in ATRT cells in response to IGF-I stimulation, we initially used RTK array as a screening method for activated growth regulatory pathways, that can potentially cross-talk with IGF-IR and ultimately enhance the growth and survival of these cells. Results clearly indicated that IGF-I induces phosphorylation of VEGFR-2. Many studies have provided evidence for the role of VEGFR-2 in tumor vascularization, growth, and metastasis. We then validated the results in both BT12 and BT16 cell lines by Western blot. The downstream signalling molecule ERK1/2 was examined, which is functional in the IGF-IR and VEGR-2 pathways, as well as in multiple other signalling pathways. Thus, the IGF-I signalling experiments do not strictly indicate that IGF-I induces activation of one particular pathway. However, the IGF-I signalling experiments show for the first time that cross-talk occurs between IGF-IR and VEGFR-2.

(b) IGF-I stimulates VEGF secretion in ATRT cells through HIF-1α:

Recent work has shown that various growth factors and cytokines can stimulate HIF-1 α expression, thereby triggering transcription of numerous hypoxia-inducible genes by oxygen independent mechanisms. IGF-1-induced VEGF synthesis and secretion has been reported in several non-neuron cell lines, including human retinal pigment epithelial cells [120], colon cancer cells [121], endometrial adenocarcinoma cells [122], and human mesangial cells [123]. In this study, we examined whether accumulation of HIF-1α is induced by IGF-I. Our results demonstrated for the first time, to our knowledge, that that IGF-I induced a time- dependent increase in HIF-1α. More importantly, the increase in

HIF-1α expression induced by IGF-I, was accompanied by increasing levels of VEGF protein levels. We found that VEGF expression started to increase 6 h after IGF-I treatment, approached maximal expression at 12 h, and remained expressed until 24 h. The molecular mechanism by which IGF-I regulates HIF-1α transcription remains to be elucidated. Together, these results suggest the possibility of an indirect pathway for IGF-IR cross -talk with VEGFR-2 pathway through IGF-I-induced VEGF synthesis and secretion this is accompanied by a concomitant activation of VEGFR-2 signalling pathway.

(c) Cross-talk between IGF-IR and VEGFR-2 leads to IGF-IR inhibitor resistance:

Several preclinical and clinical discoveries have associated IGF-R tyrosine kinase inhibitors with antitumor activities. However, it was suggested that the presence of receptor cross-talk between IGF-IR and other pathways (EGFR, HER-2, ER and PDGFR) can mediate the resistance of cancer cells to IGF-IR tyrosine kinase inhibition therapy. In this study we showed that treating ATRT cells with AEW541 inhibits IGF-IR activation and its downstream ERK1/2. However, the presence of IGF-I provided these cells with a mechanism that compensates for the inhibition of IGF-IR by VEGFR-2 activation. This was transmitted to ERK1/2, leading to sustained cell survival signalling. These results suggest the possibility of a direct interaction between IGF-I and VEGFR-2, as these cells are known to highly express and produce IGF-I, which could overcome AEW541 effect by activating the VEGFR-2 pathway.

(d) Role of IGF-IR/VEGFR-2 cross-talk in cell migration:

IGF has been linked tumor cell migration, which is a critical step in tumor progression and metastasis. ATRT frequently presents with significant infiltration into the brainstem, making tumor resection a difficult task, which reveals the potential of aggressive ATRT cells for invasion and migration. We hypothesized that IGF-I increases ATRT cells

migration, and that the presence of a receptor cross-talk will enhance IGF-I mediated migration. In this study, we found that IGF-I induced ATRT cells movement. Furthermore, treating cells with AEW541 lead to inhibition of cells migration. We also showed that IGF-I could overcome the block on IGF-1R signalling when cells were stimulated with IGF-I in absence of other growth factors. These results suggested that IGF-I can signal through an alternative pathway other than IGF-IR pathway, in order to stimulate cells movement. The combination of IGF-1R and VEGFR-2 targeted inhibitors effectively blocked cells migration within hours in presence of IGF-I. This new finding suggests a link between the two pathways, via IGF-I mediated effect.

(e) Synergistic activity of AEW541 with VEGFR-2 inhibitors:

The generation of resistance to RTK-targeted therapeutics has been a major obstacle in the utility of this family of agents. In addition, tolerability concerns have also limited the effectiveness of single agent RTK-targeted therapies in the past. Drug combination strategies against different RTKs could improve efficacy, as exemplified by very effective combination trials targeting EGFR pathway [124]. Therefore, we hypothesized that co-targeting IGF-IR and VEGFR-2 using small molecules receptor tyrosine inhibitors will optimize cell killing, as compared with treatment using a single agent. We screened a comprehensive library of targeted therapeutic agents, using *in vitro* cytotoxicity assays of agents targeting IGF-IR and VRGFR-2 RTK inhibitors. Our current results (figure 3.2, figure 3.3, and table 3.1), as well as previously published studies, have alluded to the critical role of IGF-IR activity in ATRT cells. AEW541 is a selective inhibitor for the IGF-IR kinase activity, and is shown to inhibit tumor cell growth in a wide range of cancer types. Previous studies showed that AEW541 induced apoptosis in acute myeloid leukemia cells, and sensitized leukemic blasts to etoposide [125-126]. Therefore, we wanted to

investigate the effect of combining VEGR-2 inhibition with AEW541. In order to identify this effect, a constant amount of AEW541 at its IC_{25} concentration was added to increasing concentrations of a VEGFR-2 inhibitor, and the resulting CI were then calculated. We observed combination indices less than 1, suggesting drug synergy under these experimental conditions. We then evaluated the consequence of these combinations on apoptosis, and found that capability of AEW541 to induce apoptosis is potentiated by combined therapy, as seen by the changes in caspase-7 and -3 and PARP cleavage, as compared to treatment using a single agent. Such information is crucial for the development of future drug combination therapies, which could be used to optimize cell killing, and reducing toxicity and the potential for drug resistance.

(f) IGF-IR knockdown cells treated with IGF-I caused an increase in tyrosine phosphorylation of VEGFR-2:

The inhibition of IGF-IR expression or function has been shown to blocking IGF signalling, enhancing apoptosis and inhibiting growth and survival of many tumour types *in vitro* and *in vivo*. It is also important to circumvent potential mechanisms of resistance to novel therapies. Therefore, the knockdown study was designed to investigate whether the VEGFR-2 axis is capable of compensating for IGF-IR loss. Knockdown of IGF-IR resulted in a compensatory up regulation and phosphorylation of VEGFR-2. This was associated with enhancement of IGF-I induced signalling via ERK1/2, suggesting that IGF-I induced activation of an alternative pathway compensating for IGF1R loss.

In summary, our results showed, for the first time, a novel interaction and cross-talk between the IGF-I and VEGF receptors in ATRT cells. In this thesis, the ability of IGF-IR to promote invasive tumor growth of ATRT has been explained by different mechanisms:

(*a*) Increased production of the angiogenic vascular endothelial growth factor, via activation of IGF-IR.

(*b*) Increase in cellular motility and cellular invasiveness of ATRT cells by activation of IGF-IR.

(c) The interactions of IGF-IR with other receptor tyrosine kinases, which potentiate the biological effects of each other.

The presence of cross-talk between two potential therapeutic targets (IGF-IR and VEGFR-2) may be of particular importance to overcome acquired drug resistance, and would seem to have intriguing potential for human cancer therapy. Drugs that are clinically applicable against IGF-IR have been developed, and inhibitors targeting VEGFR-2 are emerging. The strategy of targeting both receptors simultaneously provided synergistic effects with enhanced induction of apoptosis. However, it is still unclear whether IGF-IR and VEGFR-2 can directly transactivate each other or cross-talk at the level of their principal signalling intermediates. Future research will lead to better understanding of the complex biological processes underlying the interactions both receptors, and hopefully will eventually help to provide an optimized patient therapy.

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