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Insulin-Like Growth Factor Receptor Signaling in Thyroid Cancers: Clinical Implications and Therapeutic Potential

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1. Introduction

Human Thyroid Tumors represent a multistage model of epithelial tumorigenesis. Even though a majority of these tumors originate from follicular cells, they exhibit a broad spectrum with different phenotypic characteristics and variable clinical behavior. Our recent studies suggest that numerous growth factors and their receptors may be abnormally overexpressed or constitutively activated in thyroid tumors to influence their biological behavior. In this chapter we review our current understanding of the role of Insulin-like growth factors (IGFs) and their receptors in the pathogenesis of thyroid cancer and how expression of pIGF-IR may be an indicator of their clinical behavior [1]. Mechanistic evidence for direct involvement of IGF-I signaling in metastasis of anaplastic thyroid cancer & on tumor associated angiogenesis [2] is also discussed. Finally, as several small molecular inhibitors of IGF-IR signaling (peptide-based antagonists and monoclonal antibodies) are being tested, we will discuss the potential impact of utilizing IGF-I signaling pathway as a therapeutic target [2, 3] for aggressive thyroid cancers especially in cases where the current therapeutics have failed to show a favorable outcome.

Thyroid cancer (TC) is one of the most common endocrine malignancies worldwide. According to recent American Cancer Society (ACS) estimates, its incidence is rising in the US, with an average increase of 6% between 1975 & 2008[4]. Recent data further indicates that its incidence is three times higher in women than in men amongst all ethnic populations. Even though a majority of these tumors originate from follicular cells, they exhibit a broad spectrum with different phenotypic characteristics and variable clinical outcomes. Histopathological evaluation of TC specimens suggests that these tumors can be further sub-classified as differentiated thyroid carcinoma (DTC), undifferentiated (anaplastic) thyroid carcinoma (ATC) and medullary thyroid carcinoma (MTC). DTC and ATC are also referred to as nonmedullary thyroid cancer (NMTC) and may include subtypes like Hurthle cell carcinomas (HCC). Ninety to ninety three percent of all thyroid tumors are of differentiated phenotypes and have a papillary (PTC) or follicular (FTC)

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morphology. Usually these tumors follow a protracted clinical course with a 10-year survival rate of 92%. Another 5% are MTCs where the 5-year survival rate is approximately 50%. On the contrary, ATCs arise either de novo or may evolve from follicular or papillary carcinomas. These tumors are rare, occurring in only 1-2% of all TCs, and are invariably associated with fatal outcomes [5]. In the clinic, these patients present themselves with widely invasive local disease and are surgically incurable. The survival rate for ATCs which frequently metastasize to distant sites, is <10%. Severity of ATC is further underscored by the fact that even when these patients receive aggressive multimodal therapy such surgery, radiotherapy, and chemotherapy, more than 80% of patients die within months, especially those who are more than sixty years of age[6]. An in-depth understanding of thyroid cancer biology is therefore a necessary prerequisite to develop better management schemes for different types of thyroid cancers. Furthermore, the information gleaned could be utilized to make therapeutic advances that may improve the outcomes of thyroid cancer patients in the near future.

So far, study of genetic and epigenetic alterations in thyroid cancer cells has given us exciting new insights into the mechanisms that give rise to thyroid tumors [7]. Even though

Single TKIs	Target	Ref
gefitinib[67]	EGFR	Pennell et.al.
imatinib (STI571) [64]	c-kit	Ha et.al.
Multikinase inhibitors	**Target**	**Ref**
sorafenib[63, 66]	BRAF, VEGFR 1 and 2 and PDGFR, c-kit, Flt-3	Duntas et.al Lam et.al.
sunitinib (SU11248) [60, 61]	Flt-3, c-kit, VEGFR, and PDGFR	Broutin, et.al. Carr et.al.
Axitinib(AG-013736)[62]	All VEGFRs	Cohen et.al.
Motesanib (AMG 706)[69]	VEGFR 1–3, RET and c-KIT	Rosen et.al.
Vandetanib (ZD 6474)[68, 70]	VEGFR 2 and 3, RET and EGFR	Robinson et.al. Wells et.al.
Pazopanib (GW786034)[59]	VEGFR 1–3 and c-KIT	Bible et.al.
XL184 (Exelixis)[65]	RET, c-MET and VEGFR 1 and 2	Review by Kapiteijn et.al.
PLX 4032[65]	V600E mutant BRAF kinase, not wild type	Review by Kapiteijn et.al.
E7080[65]	VEGFRs, c-KIT and PDGFRß stem cell factor	Review by Kapiteijn et.al.

Table 1. Single and multi-kinase inhibitors under clinical development for treating thyroid cancer.

we may be far away from a clear understanding of the complete set of molecular events that transform benign thyroid cells into tumorigenic cells, a vast majority of literature indicates that signals originating from growth factors (GF) and their receptors play an important role in fueling the growth of aggressive cancer.

It is well established that GF signaling is required for maintaining the malignant phenotype through alteration of the cell cycle, induction of apoptosis, and modulation of the behaviour of tumor cell or its micro-environment [8]. It is no surprise then that GF and their receptors have become attractive candidates for targeted therapy of cancer[9]. Constitutive signaling through the receptor tyrosine kinases (RTKs), particularly the epidermal growth factor receptor (EGFR, erbB1), Her2/neu (c-erbB2), and vascular endothelial growth factor receptor (VEGFR) has been reported in multiple tumor types including TC. This has opened up the possibility of blocking them with small molecule tyrosine kinase inhibitors (TKIs) either as single agent or as a cocktail of multi-kinase complexes (see Table 1), or with human or humanized monoclonal antibodies (mAb) (see Table 2).

Trade name	Type of Ab	Target	Clinical Use
Trastuzumab	hmAb	against the juxtamembrane region of HER2	HER-2 over-expressing breast cancer
Cetuximab	mAb	competitive antagonist for EGFR	CRC, HNSCC
Bevacizumab	recom hmAb	specific for the VEGF A	CRC, NSCLC

Table 2. Monoclonal antibodies as inhibitors of receptor tyrosine kinases.

Insulin-like growth factor–I receptor (IGF-IR) is another candidate gene that has gained popularity as a viable RTK target in the last two decades for several different reasons [10, 11]. The most significant reason is that multiple oncogenes require the presence of IGF-IR to achieve cellular transformation[12]. In addition, IGF-I signaling confers resistance to many therapies that currently constitute the standard of care in oncology[13-15]. Furthermore, epidemiological studies have shown that elevated plasma levels of IGF-I are associated with higher risk of several cancers (breast, colon, prostate and lung) [16-19]. All of these data suggest that instead of using conventional cytotoxic chemotherapy, targeting the IGF-I axis may be an important, effective and well-tolerated therapeutic alternative for treating cancer[20]. Indeed, several anti-IGF-IR compounds are in Phase I and Phase II clinical trials[21] to measure their anti-tumor effects as single agents or when given in combination with chemotherapy or radiotherapy (Table 3). However, very few studies have looked at the role of IGF-IR signaling in TC or evaluated the potential of anti-IGF-IR therapy for in this cancer. We investigated whether enhanced IGF-IR signaling promotes

class	ID	Company	Phase of dev	Target organs
IGF-IR inhibition	IGF-IR specific Antisense oligonucleotides			
IGF-IR inhibition	IGF-IR specific Si or ShRNA			
IGF-IR specific Antibodies				
	CP-751,871 (figitumumab)	Pfizer	Phase III	NSCLC, and ovarian carcinoma, prostate, breast, colon
	IMC-A12	Imclone Systems	Phase II	neuroendocrine tumors, prostate, colorectal, breast, pancreatic, liver, and head and neck, HCC
	AMG 479 (ganitumab)	Amgen	Phase II	Ewing's/PNET, colorectal, breast, pancreatic, and small-cell lung cancer
	MK0646 (dalotuzumab)	Merck	Phase II	Colon, Pancreas, neuroendocrine tumors
	AVE1642	Sanofi_Aventis	Phase II	Breast, Liver, Multiple myeloma, Ewing's sarcoma
	R1507	Roche	Phase II	Lung, breast, sarcoma, advanced solid tumor
TKI	NVP-AEW541	Novartis	Phase I/II	Multiple myeloma, glioblastoma, Ewing's sarcoma and breast cancer
	NVP-ADW742	Novartis		
	NVP-TAE226	Novartis		
	BMS-536924	Bristel Myers Squibb		
	BMS-554417*	Bristel Myers Squibb		
	BMS-754807*	Bristel Myers Squibb		Breast, head and neck, advanced solid tumor
	OSI-906 (PQIP)	OSI Pharma	Phase III	Adrenocortical, ovarian, breast, advanced solid tumor, colon and liver
	A-928605	Abbott		
	XL-228**	Exelixis	Phase I	CML, lymphoma, cancer
	AXL1717	Axelar AB	Phase I/II	
Antibodies for the ligands	KM1468 KM3168 KM3002	Kyowa Hakko	preclinical	
IGFBPs	Recombinant human IGFBP3 protein SomatoKine/IPLEX	Imsmed Incorporated	Phase I/II	Myotonic dystrophy type 1
	cyclolignan picropodophyllin (PPP)	Biovitrum		
	nordihydroguaiaretic acid***	INSMED		

*Targets IR in addition to IGF-IR, **targets Abl, SFK,Src, Aurora kinase A, *** targets Her2

Table 3. Drugs targeting Type I insulin-like growth factor receptor.

thyroid cancer progression and if so, is it a viable candidate for RTK therapy in thyroid cancer. In this chapter, we review the basics of IGF-IR signaling, our experience with over-expression of IGF-IR signaling components in TC and how blockade of IGF-I signaling through its receptor has the potential to curb the growth of poorly differentiated FTC and ATC. Later sections of the chapter also describe important molecular changes resulting from IGF-IR blockade and their influence on tumor growth.

2. Brief overview of insulin-like growth factor receptor signaling

Major components of IGF signaling axis include the three ligands (IGF-I, IGF-II & Insulin), IGF binding proteins (IGFBPs) and the three tyrosine kinase receptors, namely, IGF-IR, IGF-IIR and Insulin receptor (IR). All of these ligands can act as circulating hormone or tissue growth factors. Similarly, the IGFBPs are also produced in the liver or other organs and

Fig. 1. Schematic of Insulin like growth factor receptor illustrating its different domains and the position of sulphide bonds. IGF-IR is a trans-membrane tyrosine kinase and consists of two αβ chains and has high affinity for its ligands IGF-I and IGF-II. Insulin can also bind and signal through this receptor although with much lower affinity.

delivered to the target tissue in an endocrine manner. The balance of IGF-I, either bound to IGFBP or its unbound form determine whether a cell will follow a survival pathway or follow an apoptotic course. Free IGF-I exerts its effects through the activation of IGF-IR, its preferred cell surface receptor. IGF-IR is synthesized as a precursor peptide of 1367 amino acid residues. It is then cleaved at residue 706, to dissociate the α chain containing the extracellular domain from the β-chain that encompasses the transmembrane and tyrosine kinase domains (Fig. 1).

It moves to the membrane fully assembled in the dimeric form with two α-chains and two β-subunits[22]. Signaling through IGF-IR is initiated when IGF-I and IGF-II produced by the liver and at many extra hepatic sites including tumor cells and stromal fibroblasts or insulin bind to IGF-IR. Upon ligand binding, IGF-IR is auto phosphorylated to stimulate its tyrosine kinase activity that subsequently phosphorylates additional intracellular substrates, including insulin receptor substrates-1 through 4 (IRS-1-4) and Shc (an SH2 domain containing adaptor protein). These early events activate multiple signaling pathways, including the mitogen-activated protein kinase [MAPK, extracellular signal-regulated kinase (ERK)] and phosphatidylinositide 3-kinase (PI3-K)/Akt-1 (protein kinase B) pathways (Fig. 2)[23, 24]. Signaling from IGF-IR is known to play a crucial role in organ development[25-27]during embryogenesis and regulation of mitogenesis through suppression of apoptosis and stimulation of proliferation[28].
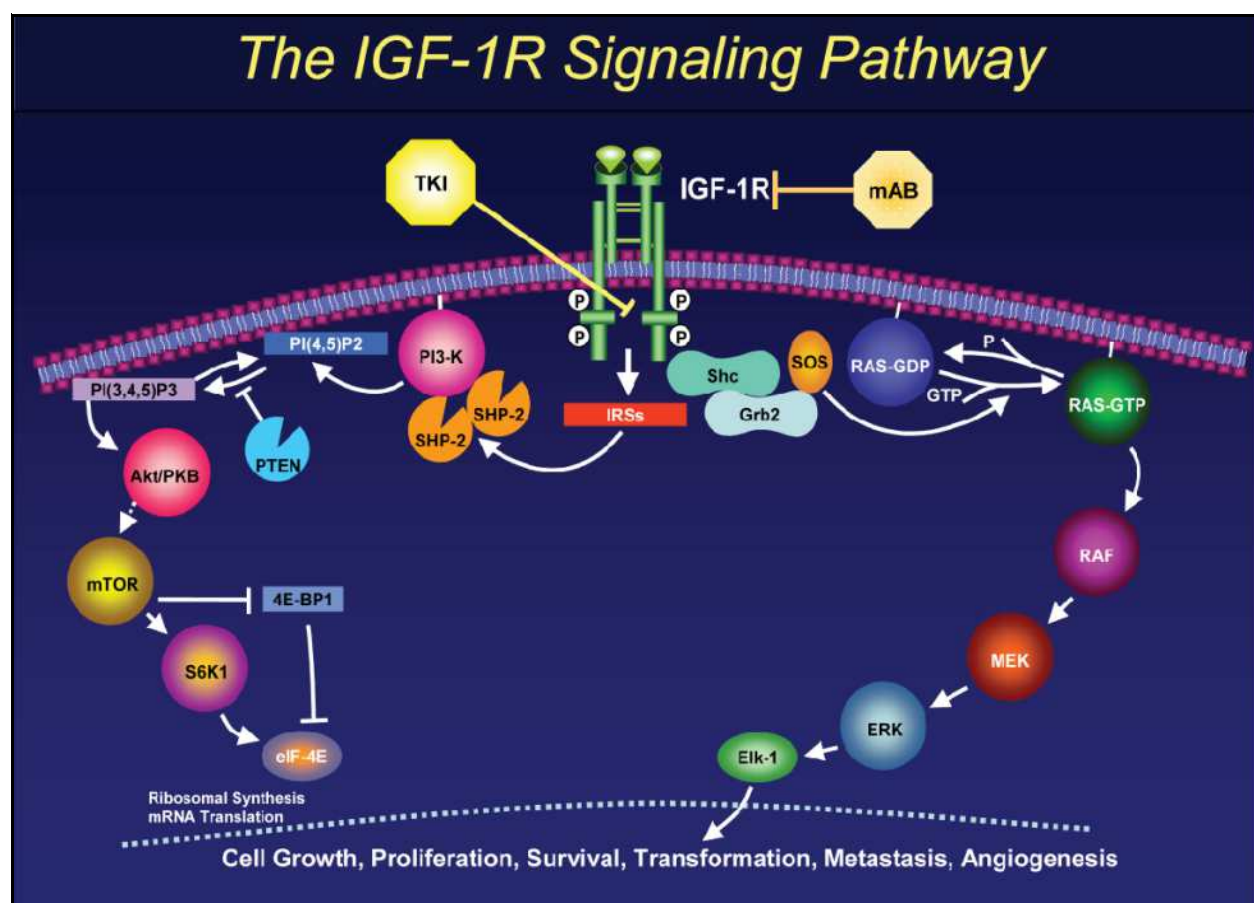

Fig. 2. IGF-IR signaling and two most frequently used IGF-IR inhibition strategies: In response to ligand binding, IGF-IRβ gets activated and phosphorylates adaptor proteins belonging to the IRS family or SHC. Activation of IRS and SHC leads to activation of extracellular signal–regulated kinase (ERK) 1/2 of the MAPK cascade via the growth factor

receptor binding protein 2 (Grb2)/Sos/Ras/Raf/MAPK extracellular signal–regulated kinase kinase (MEK) pathway. IRS proteins also bind to the p110 subunit of PI3K, leading to the generation of phosphatidylinositol 3,4.5-triphosphate (PIP3) and phosphorylation of Akt by phosphoinositide dependent kinase (PDK1). Phosphorylation of Akt leads to subsequent activation of mammalian target of rapamycin (mTOR), eukaryotic translation initiation factor 4E (eIF4E), and p70S6 kinase (S6K). Activation of these downstream signaling pathways leads to enhanced proliferation, survival, and metastasis in cancer cells. Similar signaling pathways are activated by IR and other IGF-IR/IR hybrid receptors.

In normal cells, the IGF/IGF-IR signaling is regulated at multiple levels (Fig. 3)[29]. Initially, Growth hormone-releasing hormone (GHRH) stimulates the expression of growth hormone (GH) produced in the pituitary gland. GH then stimulates the secretion of IGFs and IGFBPs from the hepatocytes [30]. As stated earlier, activation of IGF-IR is tightly regulated by amount of free forms of the ligands, which is controlled by the action of IGFBP and the type 2 IGF receptor (IGF-IIR) also known as mannose 6-phosphate receptor. This receptor can bind IGF-II but lacks tyrosine kinase activity. Accordingly, when IGF-II binds the receptor, it fails to transduce its signal and just serves as a sink for the IGF-II. This reduces the circulating levels of IGF-II thus enhancing the signalling of IGF-IR [31].
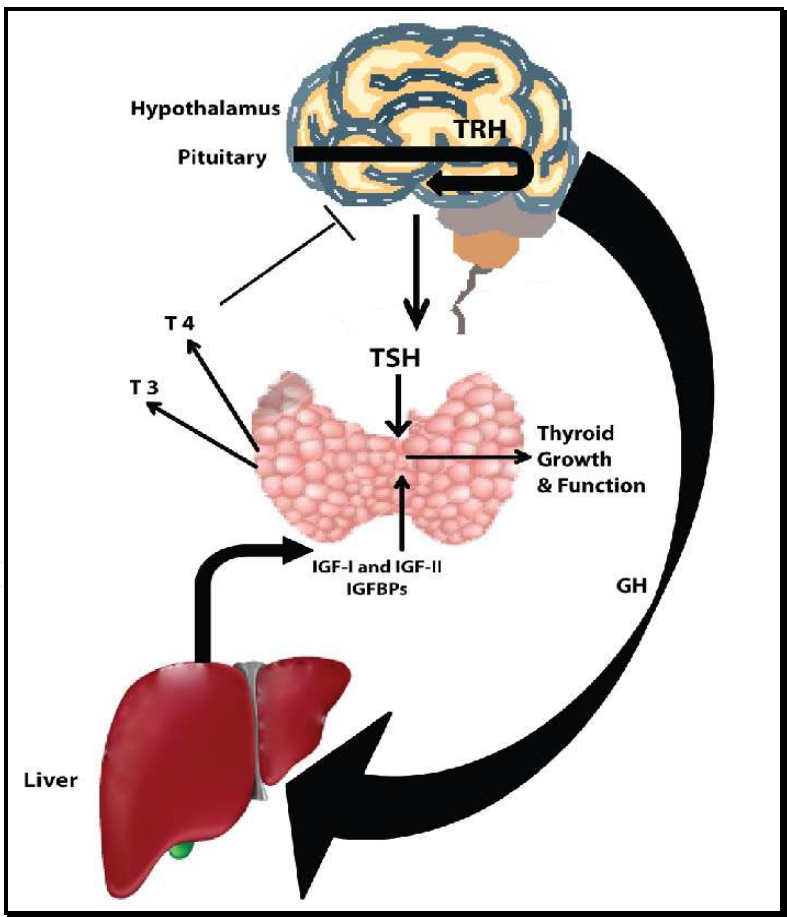

Fig. 3. Growth hormone (GH) and Thyroid stimulating hormone (TSH) are produced under the influence of hypothalamic factors. GH controls the secretion of IGF-I, IGF-II and IGFBPs from liver, whereas TSH controls the secretion of T3 & T4 that regulate thyroid growth. In normal thyroid cells TSH cooperates with IGF-I signaling to promote thyroid growth and

function. However, in some cases, excessive constitutive synthesis of IGF-I may be involved in abnormal growth of the thyroid.

In contrast, IGFBP 1-6 modulate IGF activity by reducing bioavailability of IGFs that may bind to the IGF-IR. The complex balance between the IGFs and the IGFBPs is modulated by specific IGFBP proteases, such as matrix metalloproteinase (MMP)[32]. Like IGF-I and IGF-II, Insulin can also signal through the IGF-IR, IR and the IGF-IR/IR hybrid receptor to induce a variety of biological effects in typical insulin target (adipocyte, hepatocyte, and myocyte) cells[33] and in cancer cells. Upon ligand binding, insulin/IGF-IR undergoes auto-phosphorylation on tyrosine residues, activating the same downstream signaling cascade as the ones initiated upon IGF-I binding. However, as insulin binds to the IGF-1R with 100- to 1000-fold lower affinity than IGF-I[34], most of the signaling from the IGF-IR may be assumed to be the result of IGF-I signaling. Nevertheless, the high degree of homology of IGF-IR to insulin receptor has been a considerable challenge for the development of anti-IGF-IR therapies that are specific to IGF-IR.

3. Role of IGF-IR signaling axis components in human thyroid cancers

Inappropriate IGF-IR signaling is implicated in the development and progression of several human malignancies [35] including those of the thyroid[36-38], and often correlates with poor prognosis[39, 40]. Activation of IGF-IR is essential for the mitogenic effects of TSH and for thyroid function [41-44]. Unlike other growth factor receptors, IGF-IR and insulin receptor (IR) are not inhibitory to thyroid function. Instead they cooperate with TSH to stimulate growth (Fig. 3). However, in some cases, excessive constitutive synthesis of IGF-I has been shown to be involved in abnormal growth of the thyroid[45]. Additional accumulated evidence from studies of other neoplasms suggests that in addition to the TSH-IGF-I nexus, there are several other mechanisms by which IGF-IR signaling may be dysregulated in human tumors. It can be constitutively activated through autocrine or paracrine signaling[39, 46]. Alternatively, ligand-independent mechanisms can result in the activation of the receptor[47]. By far, the most common occurrence is overexpression of IGF-IR. However, whether that is the case in thyroid cancer is unknown.

4. IGF-I and IGF-IRß expression is high in all histological subtypes of thyroid cancer and thyroid cancer cell lines

In recent studies, the authors measured the expression of IGF-I, IGF-IRß and phosphorylated IGF-IRβ (pIGF-IRβ) in normal and neoplastic human thyroid tissue to determine whether IGF-I axis plays a role in thyroid tumor progression. Evaluation of the distribution and abundance of IGF-l in human thyroid cancers with different histopathologic characteristics showed that immunoreactive IGF-l was present in all the thyroid tissues examined. Its expression was lowest in normal the thyroid tissues and highest in all thyroid carcinomas studied. Examination of expression level of IGF-IRβ in normal and neoplastic thyroid tissue on human tissue arrays showed that none of the normal thyroid tissue specimens stained positively for IGF-IRβ, whereas 60 out of 63 specimens of thyroid cancer were positive. The intensity of staining ranged from + to +++ (+ being low, ++ moderate and +++high). Compared to the normal thyroid tissue specimens, the positive staining rate of ATC (37/39, 94%) FTC (11/11, 100%) and PTC (12/13, 92%) specimens revealed statistically significant differences in IGF-IRß expression

(P < 0.001). No statistical differences between ATC, FTC-and PTC-positive staining (Fig. 4A and Table 4) were noted.

Similar analysis of the thyroid cancer cell lines confirmed our findings from human tumor specimens. As per our expectation, most of the FTC and PTC cell lines showed little variability in IGF-IRβ expression. But its expression was comparatively lower in ATC cell lines. pIGF-IRβ expression on the other hand was variable. Overall, FTC and PTC cell lines had higher pIGF-IRβ levels compared to the normal thyroid cells or the anaplastic cell lines (Fig. 4B & C). It is important to mention here that the data presented is for representative cell lines only. More details can be accessed at Wang et.al. (2006)[2]. Nevertheless, despite the high IGF-IR content of several of the thyroid cancer cell lines tested, auto-phosphorylation of the receptor in response to IGF-I stimulation was observed only in cell lines that had an intact IGF-I axis. Evaluation of the phosphorylated form of the receptor (pIGF-IR) in surgical specimens of human FTC, PTC and ATC indicated that both FTC and PTC specimens had moderate to high levels of pIGF-IR. But neither the ATC specimens nor most of the ATC cell lines tested had detectable levels of pIGF-IR (Fig 4B). This data implied attenuated expression of growth-signaling components of the IGF system. In particular low pIGF-IR expression may be associated with malignant phenotype or more aggressive form of thyroid cancer. To test this hypothesis further, a quantitative immunohistochemical assay for pIGF-IR expression was developed[1] and archival human thyroid tumor microarrays containing specimens with 10 to 12 year follow up were analyzed as described.

| Tissue type | No. of samples | IGF-IRß staining | | | | Positive |
| | | Level of staining | | | | staining (%) |
		0	+	++	+++	
Normal thyroid	8	8	0	0	0	0
Follicular thyroid cancer	11	0	0	4	7	100.0
Papillary thyroid cancer	13	1	7	4	1	92.3
Anaplastic thyroid cancer	39	2	12	15	10	94.9

Table 4. Level of immunohistochemical staining for IGF-IRβ in normal thyroid, follicular, papillary and anaplastic thyroid cancer tissue from 63 human surgical samples (including specimens on tissue array and permanent pathological slides) [2].
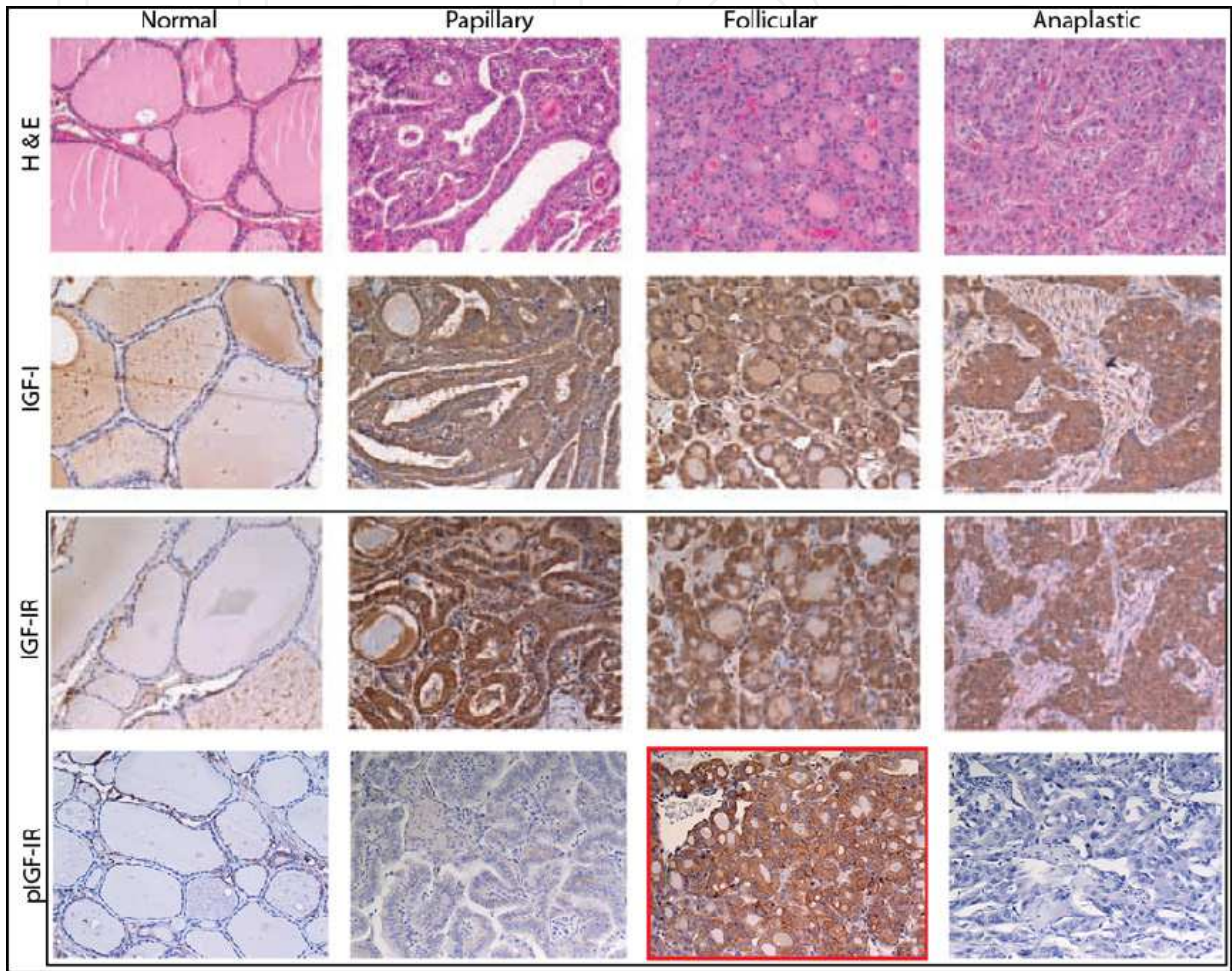
Fig. 4A. Expression of pIGF-IRβ is down regulated in majority of anaplastic and some papillary thyroid cancer specimens: Immunoperoxidase staining of thyroid tumor tissue sections to visualize expression of endogenous IGF-I, IGF-IRβ and pIGF-IRβ. The black boxed area indicates that as compared to normal thyroid tissue, PTC, FTC and ATC specimens show overexpression of IGF-IRβ, whereas expression of pIGF-IRβ is down regulated in majority of ATC and PTC specimens. In contrast pIGFIRβ expression (highlighted in red boxed specimen) is retained mostly in FTC specimens [1].

Fig. 4B. & C. IGF-IRβ/IRβ and pIGF-IRβ expression in representative thyroid cancer cell lines detected immunochemically using Western blotting (B) and Immunofluorescence (C). Total IGF-IRβ expression was detectable in all cell lines tested. pIGF-IRβ expression varied amongst cell lines and was almost undetectable in ATC cell lines. Its expression was lower in normal thyroid (TAD2) cells compared to the FTC cell lines. C) Immuno-fluorescent detection of pIGF-IRβ in FTC and ATC cell lines after stimulation with 10ng/ml IGF-I (top panels) or in serum free media (SFM). Green signal = pIGF-IR; Blue = DAPI.

4.1 pIGF-IR expression is high in differentiated thyroid cancers but its expression is attenuated in more aggressive thyroid cancers

Two thyroid tumor tissue arrays (TMA) containing 120 specimens on one and 84 specimens on the other, were analyzed in this study. One of the arrays also contained six pairs of normal thyroid tissue from the same patient. Detailed analysis of these tissue arrays (see Table 6 for demographics) confirmed that the pIGF-IR content of the differentiated component of the tumors was higher as compared to the matched pair of the normal tissue specimen. However, on the whole, all poorly differentiated tumor types, particularly the ATCs, showed negligible expression of pIGF-IR (Fig. 5).
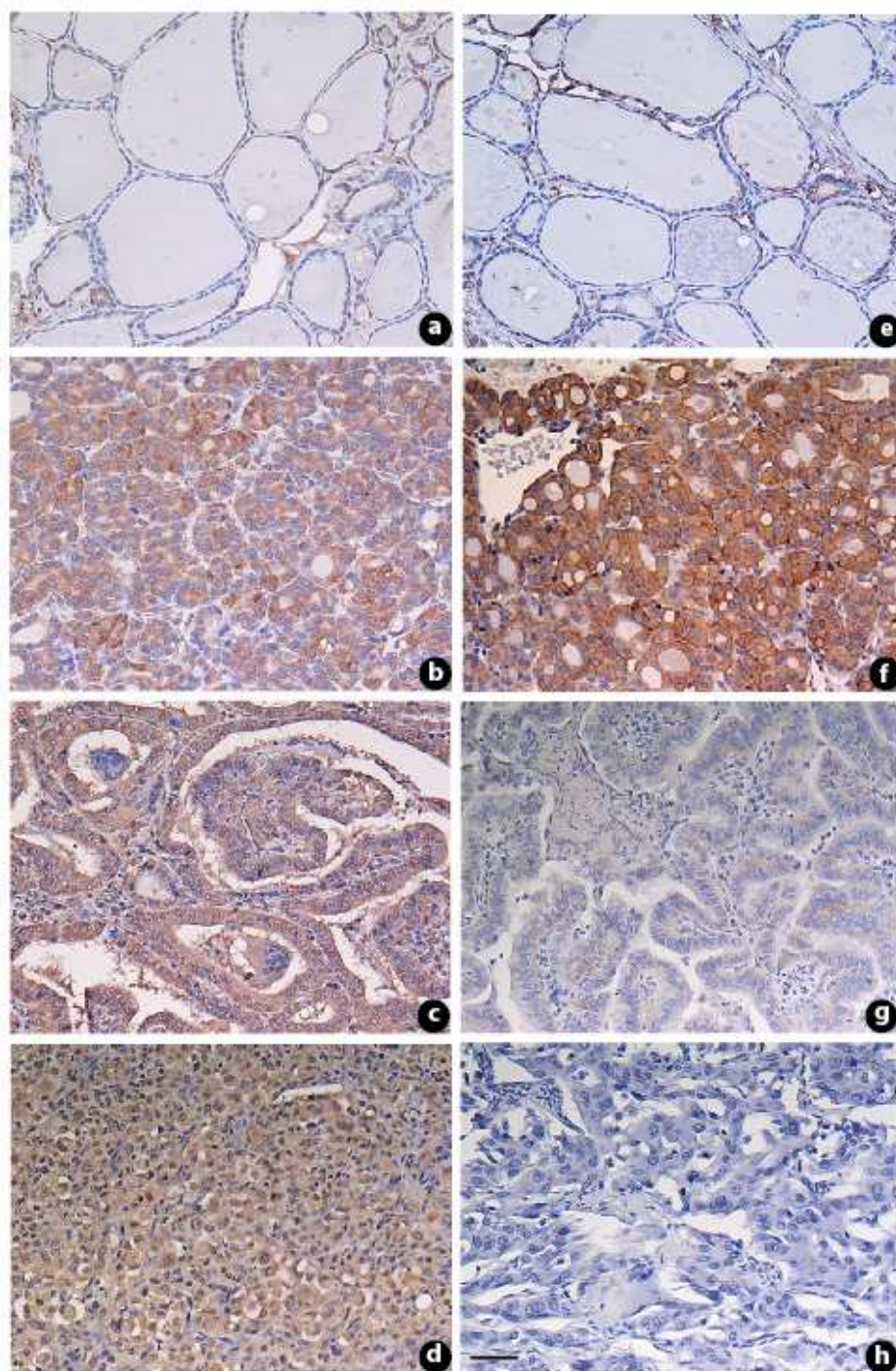
Fig. 5. Immunohistochemical analysis of IGF-IRβ and pIGF-IRβ/IRβ in histological subtypes of thyroid carcinoma: **A.** Representative paraffin-embedded sections of normal and histologcal subtypes of thyroid carcinoma treated with the anti-IGF-IRβ antibody (a – d) and anti-pIGF-IRβ/IRβ Ab (e-h). Panels a & e are sections from normal thyroid tissue treated with the two antibodies mentioned above. Note that the normal tissue has very low levels of IGF-IR or pIGF-IR/IR as compared to the adjacent panels showing intense staining in tumor tissues. Additionally, only follicular thyroid carcinoma samples and few papillary carcinoma were more often positive for anti-pIGF-IRβ/IRβ antibody staining even though all the tumor tissue types had detectable IGF-IRβ expression. Scale bar represents 50μm [1].
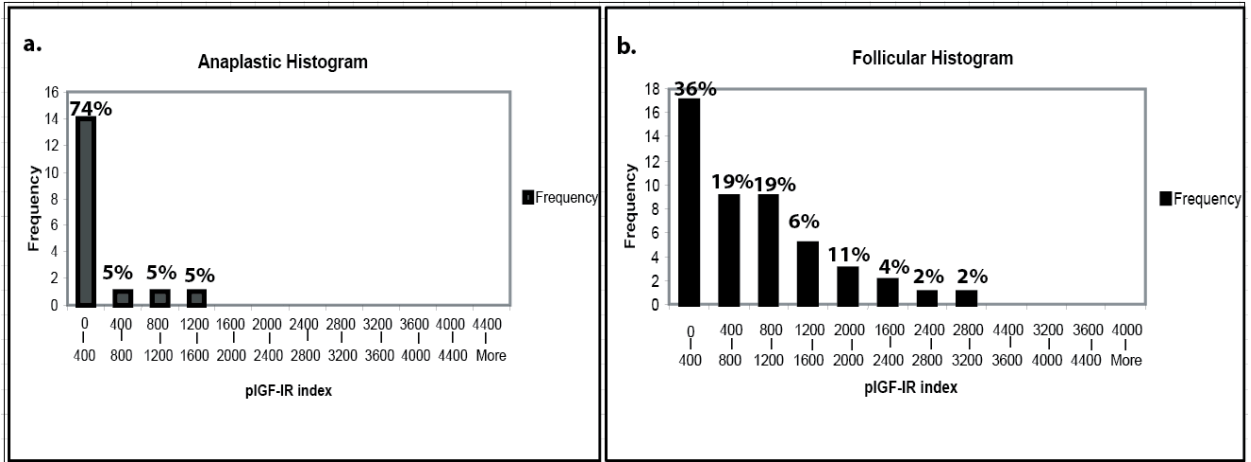
Fig. 6A. pIGF-IRβ/IRβ index of thyroid cancer specimens: Frequency distribution of pIGF-IR/IR index in a total of 17 Anaplastic (a) and 47 Follicular (b) thyroid carcinoma cases. Overall, more FTC specimens demonstrated a higher pIGF-IRβ index.

This was further substantiated by histogram analysis of all the morphological histotypes of thyroid cancer, where 74% of ATC specimens showed a pIGF-IR index below 400 as opposed to only 34% of the FTC (Fig. 6A). Furthermore, a significant difference was noted in the median pIGF-IR index of different histological subtypes of thyroid cancer (P<0.001) (Fig. 6A). When all thyroid cancers were stratified as differentiated [FTC, PTC and Hurthle cell carcinoma (HCC)] or other thyroid cancers (ATC and MTC), the median pIGF-IR index of differentiated thyroid cancer was significantly higher than the median index of other thyroid cancers (114 vs. 63, P<0.001, Fig. 6B).
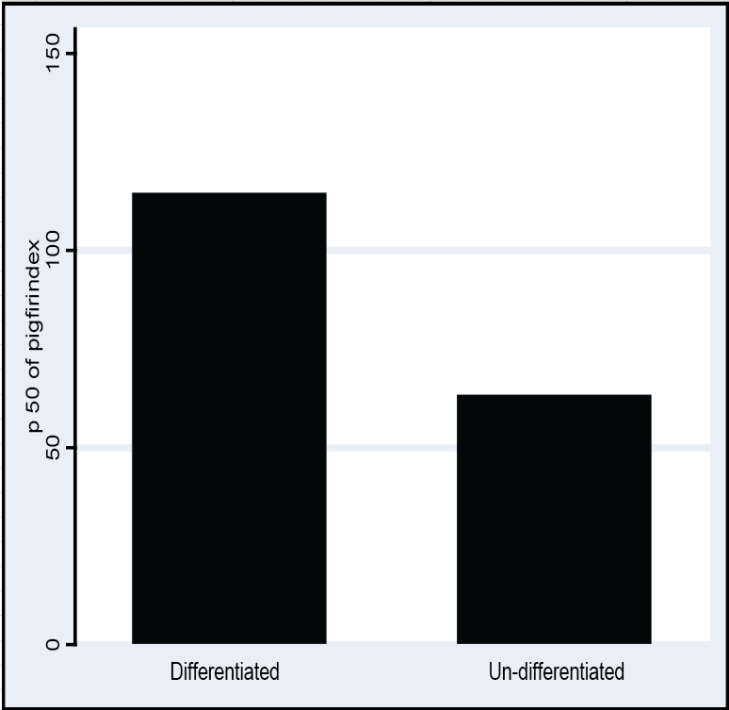

Fig. 6B. Median pIGF-IRβ/IRβ index of differentiated thyroid carcinoma was significantly higher than that of all other thyroid cancers p<0.001 (Mann-Whitney test).

To make the predictive power of our analysis more significant, additional parameters such as age, tumor size, tumor grade and lymph node metastasis were analyzed along with the pIGF-IR index of the specimen.

Our data showed that when patients with PTC or FTC were stratified according to their age, the mean pIGF-IR index of differentiated thyroid cancer patients above 45 years of age was significantly lower than the mean pIGF-IR index of patients below 45 years of age. However, due to the small sample size of our study, statistical significance couldn't be reached, although, a clear trend was noted (Table 5A). No significant difference was noted in the pIGF-IR index of tumors grouped by size or stage. Furthermore, although lymph node (LN) metastasis is not a good prognostic indicator in thyroid cancer, it does indicate recurrence and local control. Accordingly, when tumors were stratified based on their LN status, our analysis indicated that patients with differentiated thyroid cancer without lymph node metastases have a significantly higher pIGF-IR index (P = 0.03) as compared to patients with lymph node metastasis. Once again, no significant difference was noted among those patients with poorly differentiated thyroid cancer with or without lymph node metastasis (P = 0.12) (Table 5B)[1].

	Age	Median pIGF-IR	Mean pIGF-IR	P value
Follicular	<45 yrs (7)	948	1,273	0.84
	>45 yrs (10)	708	794	
Papillary	<45 yrs (7)	912	341	0.25
	>45 yrs (4)	64	59	
Differentiated	<45 (14)	991	807	0.71
	>45 (19)	125	451	

Table 5A. pIGF-IRβ/IRβ index variability by age in differentiated thyroid cancers.

Patient group	n	Median pIGF-IRß index	P value
Differentiated thyroid cancer with no lymph node metastasis	21	471.3	
Differentiated thyroid cancer with lymph node metastasis	12	78.51	0.0329*
Poorly differentiated thyroid cancer with no lymph node metastasis	7	37.5	
Poorly differentiated thyroid cancer with lymph node metastasis	15	69.07	0.1208

Table 5B. pIGF-IRβ/IRβ expression vs. patient outcome

Overall, low pIGF-IR expression was found to correlate with aggressive human thyroid carcinoma. It is thus likely that IGF-IR signaling may not be needed for progression of ATC as other cell signaling pathways may be activated in these cells thereby obviating the need

for intact IGF-IR signaling. Nonetheless, as both IGF-IRβ & pIGF-IRβ were up-regulated in a majority of differentiated thyroid carcinomas, we hypothesized that IGF-IR may be a viable potential target for therapy in patients with more differentiated types of thyroid cancer.

	Specimens	Controls	statistically analyzed
Total number in study	126	6	58
Tumor histology	Specimens		Gender
Anaplastic	19		8 (4M, 4F)
Follicular	47		18 (7M, 11F)
Papillary	12		11 (1M, 10F)
Medullary	22		14 (4M, 10F)
Other (Hurthle & Sarcomatoid)	26		7 (5M, 2F)
			M: Male
			F: Female

Table 6. Demographics of thyroid cancer patient samples used in statistical analysis of pIGF-IR index of thyroid tumor microarray (TMA)

5. Targeting IGF-IR in thyroid cancer

Targeted therapies consisting of human monoclonal antibodies against IGFBPs and the IGF-IR, or, the small molecular tyrosine kinase inhibitors (SMTKI) that target the kinase domain of IGF-IR, are a new class of pharmacological agents that have been shown to be useful in the treatment of cancers with enhanced expression and activity of IGF-IR. Pharmacological inhibition of IGF-I signaling with these therapeutic agents has been shown to significantly decrease migration, invasion, metastatic spread, and angiogenesis with little toxicity in mouse tumor models. Similarly, inhibition of IGF-IR signaling has also been reported to sensitize cancer cells to radiation and chemotherapeutic agents[48-58]. Our objective therefore, was to build upon this knowledge and investigate whether targeted therapy directed at the IGF-IR, and given in combination with chemotherapy, can be an attractive new treatment strategy for thyroid cancer[20]. Given our findings on up-regulation of components of the IGF-I signaling pathway in thyroid cancer cell lines and in human thyroid carcinoma tissue microarrays, we proceeded to test this hypothesis both *in vitro* and *in vivo*.

5.1 Choice of anti-IGF-IR compounds in clinical development

Several therapeutic agents that specifically inhibit IGF-1R but do not affect IR signaling are in preclinical and clinical development. Some of the strategies being used to interfere with IGF-1R signaling (a) reduction of IGF-1R expression by antisense nucleotides (b) siRNA or antisense RNA against IGF-IR (c) monoclonal antibodies and (d) tyrosine kinase inhibitors. Altogether, 30 different IGF-1R targeting agents are in preclinical or clinical development [59-70] (Table 3) and at least 58 active clinical trials are evaluating anti-IGF-1R targeting agents alone or in various combinations (www.clinicaltrials.gov). However, the two main strategies employed to inhibit this pathway are antibodies directed against IGF-1R or small molecule TKIs. Both approaches have their inherent strengths and weaknesses, particularly their specificity for IGF-IR and issues related to side effects like hyperglycemia. Whether sparing IR

is a good strategy or not is an important question to be considered as IR does play a role in IGF-I signaling and could compromise efficacy of anti-IGF-IR compounds. Nonetheless, we chose to evaluate the efficacy of A12 (a monoclonal Ab) and NVP-AEW541 (a SMTKI) in ATC and FTC respectively.

5.2 In vitro effects of anti - IGF-IR antibody A12 on signal transduction, cell proliferation and apoptosis of thyroid cancer cell lines

A12 is a high-affinity humanized monoclonal antibody that specifically binds to IGF-IR and blocks IGF-I and IGF-II signaling but does not block the binding of insulin to the insulin receptor. It has been shown to inhibit the growth of breast, colon, and pancreatic cancer cell lines, both *in vitro* and in subcutaneous tumors in nude mouse models by antibody-mediated blockade of ligand binding to IGF-IR. In a study of xenograft tumor model of breast, pancreas and colon cancer, A12 produced a marked increase in apoptosis with minimal toxicity [49].

In our studies, A12 was able to completely inhibit both IGF-I and IGF-II-induced phosphorylation of IGF-IR at high concentrations (100 nM) in TC cell lines (Fig 7). At lower concentrations (1 nM and 50 nM), it was able to inhibit the phosphorylation of IRS-I, Akt and MAPK. However, 10 fold more A12 was required to inhibit the IGF-2 mediated signaling (Fig. 7).
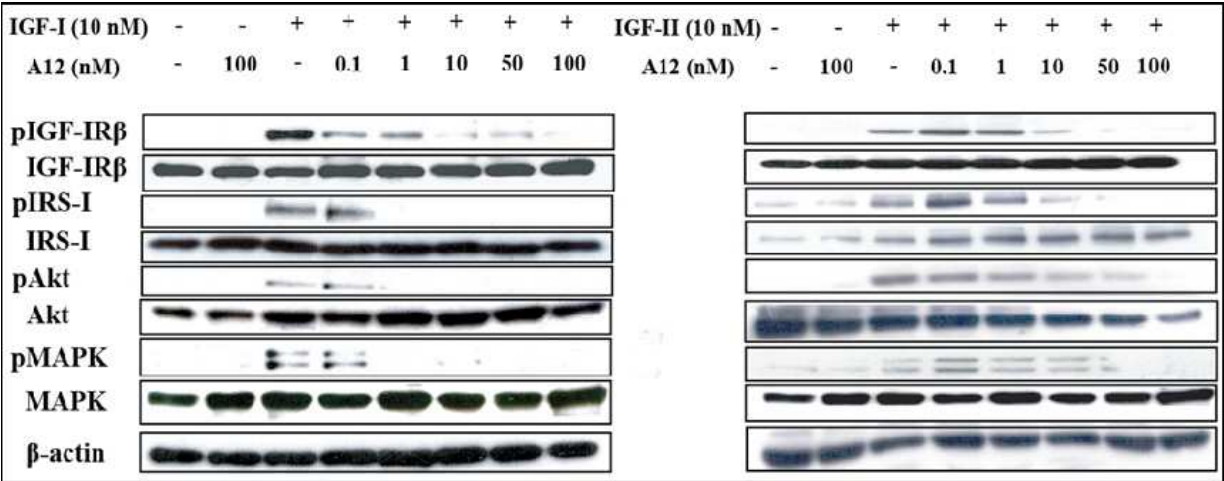

Fig. 7. Dose-dependent inhibition of IGF-1- and IGF-II-induced IGF-IRβ, IRS-I, Akt, and MAPK autophosphorylation in ARO cells after treatment with A12. Serum-starved ARO cells were treated with A12 (0-100 nM) for 2 hours and then incubated with or without IGF-I or IGF-II (10 nM) for 10 minutes. Total protein extracts were obtained to analyze IGF-IR signaling components by western blotting. Note that 10 times more A12 is required to inhibit IGF-II signaling in ARO cells [2].

Twenty-four hours after plating, the cells were treated with increasing concentrations of A12 (0-100 nM) without (A) or with (B) IGF-I stimulation for 72 hours. At the end of incubation, the inhibitory effect of A12 was measured using an MTT assay (Fig. 8).

With respect to its effect on cell proliferation, increasing the concentration of A12, with or without IGF-I, inhibited the proliferation of some TC cell lines (ARO, DRO & C643). Other TC cell lines (Hth74, KAT4 and ATC-A) were only minimally responsive to A12 treatment

(Fig. 8). To understand if this difference was due to differential induction of apoptosis, A12 treated cells were analyzed by Flowcytometry (FCM). Only minimal induction of apoptosis was observed in A12 treated cells, suggesting that growth inhibitory effects of A12 were driven through apoptosis independent pathways *in vitro*. Nonetheless, combining A12 with Irinotecan (chemotherapeutic drug of choice for head and neck cancers) was particularly useful in sensitizing ARO cells to the cytotoxic effects of Irinotecan (Fig. 9).

Fig. 8. Inhibition of survival and IGF-I induced proliferation by increasing concentration of A12 in ATC cell lines ARO, DRO, C643, Hth74, ATC-A, and KAT4. All ATC cell lines were plated in 96-well plates at a density of 2000 cells/well.

Fig. 9. Synergistic effect of A12 and Irinotecan on in vitro cell proliferation. ARO cells per well were grown in RPMI 1640 medium supplemented with 10% FBS for 24 hours. They were then treated with various concentrations of Irinotecan (0.01–15 mM) or with irinotecan with or without 5 nM A12. After a 3-day incubation period, number of metabolically active cells were determined with an MTT assay. Bars indicate mean values ± SE,. *P < .05 for cells treated with the combination versus vehicle-treated controls.

But, unlike Herceptin that down regulates ErbB2 receptor expression, IGF-IR expression remained unchanged in cells concomitantly treated with IGF-I and A12[2]. Similar findings have been reported for breast cancer cells[49].

5.3 In vivo A12 reduces tumor volume and prolongs survival in combination with irinotecan in an orthotopic nude mice model of ATC

Since it is the anaplastic thyroid carcinoma that is associated with poor clinical outcome and some ATC cell lines showed moderate IGF-IR and pIGF-IR expression [1], preclinical efficacy of A12 antibody as a single agent and in combination with chemotherapy was first evaluated in a nude mouse model of ATC[2]. Four groups [placebo (control), A12, irinotecan, and A12 plus irinotecan] of 10 mice each were analyzed. Treatment with A12 or irinotecan alone led to a 57% and 80% decrease respectively in the tumor volumes of ARO xenografts. The differences in the mean tumor volume compared with the control group were statistically significant (P = 0.023 [for A12] and 0.002 [irinotecan], respectively). However, the highest growth inhibition was achieved by the co-administration of A12 plus irinotecan. At the end of the 3-week treatment period, mice treated with A12 plus irinotecan showed a 93% decrease in the estimated mean tumor volumes compared with the control group (P = 0.001). The decrease in mean tumor volumes in mice receiving combination treatment was also significantly greater than that of the groups receiving A12 or irinotecan alone (P < 0.01) (Fig. 10A & B). A12 was well tolerated by the animals, without substantial adverse effects. The weights of the animals remained constant throughout the treatment period (data not shown) and none of the animals had to be killed before the end of the study. In the survival study, the survival rates of the mice treated with irinotecan alone and combination treatment was significantly greater than that of the mice in the control group with P values of 0.044 and 0.0003 respectively. The combination group also achieved a greater survival rate than mice treated with A12 alone (P = 0.004). However, there was no significant improvement in the survival rates between the group treated with A12 alone and the control group (P = 0.356) (Fig. 10C).

5.4 Therapeutic potential of IGF-IR specific small molecular tyrosine kinase inhibitor NVP-AEW541 in an orthotopic follicular thyroid carcinoma model

Small molecular tyrosine kinase inhibitors are another class of anti-tumor agents frequently used to inhibit IGF-IR signaling. They inhibit both ligand independent and ligand-dependent receptor phosphorylation and do not evoke immunogenic response on repeated exposure. Due to their small size, they also exhibit good tumor penetration. NVP-AEW541 (Caymen Chemical) is one such SMTKI that is a more potent inhibitor of IGF-IR (IC50 = 0.086 μM), eventhough, at high enough concentrations (IC50 = 2.3 μM) it can also inhibit the closely related insulin receptor. Structurally, NVP-AEW541 is a pyrrolo[2,3-d]pyrimidine derivative that can abrogate IGF-I-mediated survival and colony formation in soft agar at concentrations that are consistent with inhibition of IGF-IR auto-phosphorylation *in vitro*. *In vivo*, it has also been shown to inhibit IGF-IR signaling in tumor xenografts and significantly reduce the growth of IGF-IR-driven fibrosarcomas[54]. To evaluate its efficacy in thyroid cancer, human follicular thyroid cancer cell line stably expressing a constitutively activated form of IGF-IR with a downstream luciferase reporter were injected into the thyroid glands of 8-week-old athymic mice. We found that injection of 2.5×10^5 WRO cells was sufficient for
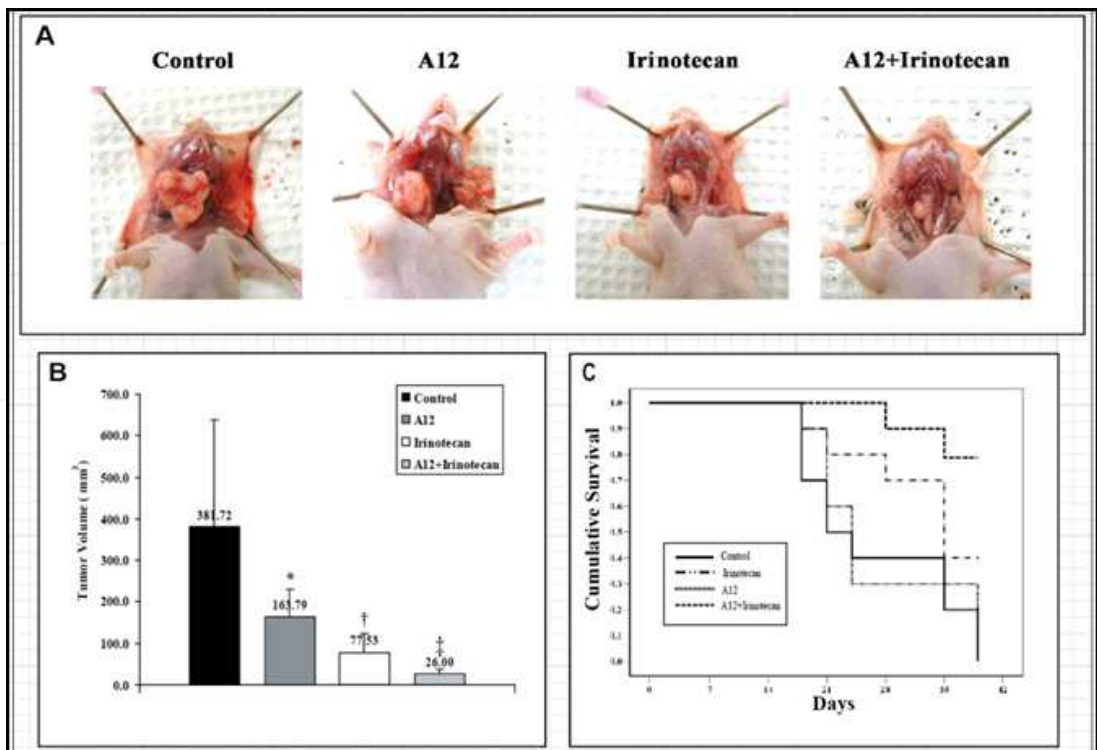
Fig. 10. In vivo antitumor effects of single drugs or simultaneous combination of irinotecan and A12 on tumor growth and survival in an orthotopic nu/nu model of ATC. ARO cells were injected into the right thyroid lobe of the mice. Four days after injection, the mice were randomized into 4 groups (10mice in each group) and the drugs were administered as follows: irinotecan (50 mg/kg i.p. every week) or A12 (1mg/250ul/mouse 2x weekly). A) After 3 weeks of treatment, all the mice were killed, and necropsy was performed. Representative images from each group of mice are shown to highlight the effect of drugs over control. B)At the end of the growth-inhibition study, the tumors were measured in three dimensions, and the mean tumor volumes were calculated in each group.* $P = 0.023$, compared with the control group; † $P = 0.002$, compared with the control group; ‡ $P = 0.001$, compared with the control group; and $P < 0.01$, compared with the A12-alone and irinotecan-alone groups (independent t-test). C, The combined treatment of A12 plus irinotecan prolonged the survival rate in the orthotopic nude mouse ATC model. Irinotecan alone or in combination with A12 significantly increased the survival rate compared with that of the control group ($P < 0.05$).

tumor development. Tumors were visualized via non-invasive real time imaging as early as 10 days post-implantation. After randomization into 3 separate groups with 10 mice in each group, they were either treated with placebo (25 mM tartaric acid solution), Irinotecan (50 mg/kg, I.P. once a week) or NVP-AEW541 (50 mg/kg PO twice daily). Real-time whole-body fluorescence imaging was carried out weekly to monitor tumor growth in response to the various treatments. After approximately three weeks, as the control group of mice became premorbid, mice from all the three groups were sacrificed and tumors harvested at necropsy. Time to premorbid condition varied between mice and was associated with primary tumor growth pattern (early local compression of the esophagus) rather than development of metastatic disease. Additionally, difficulty with oral gavage in mice with
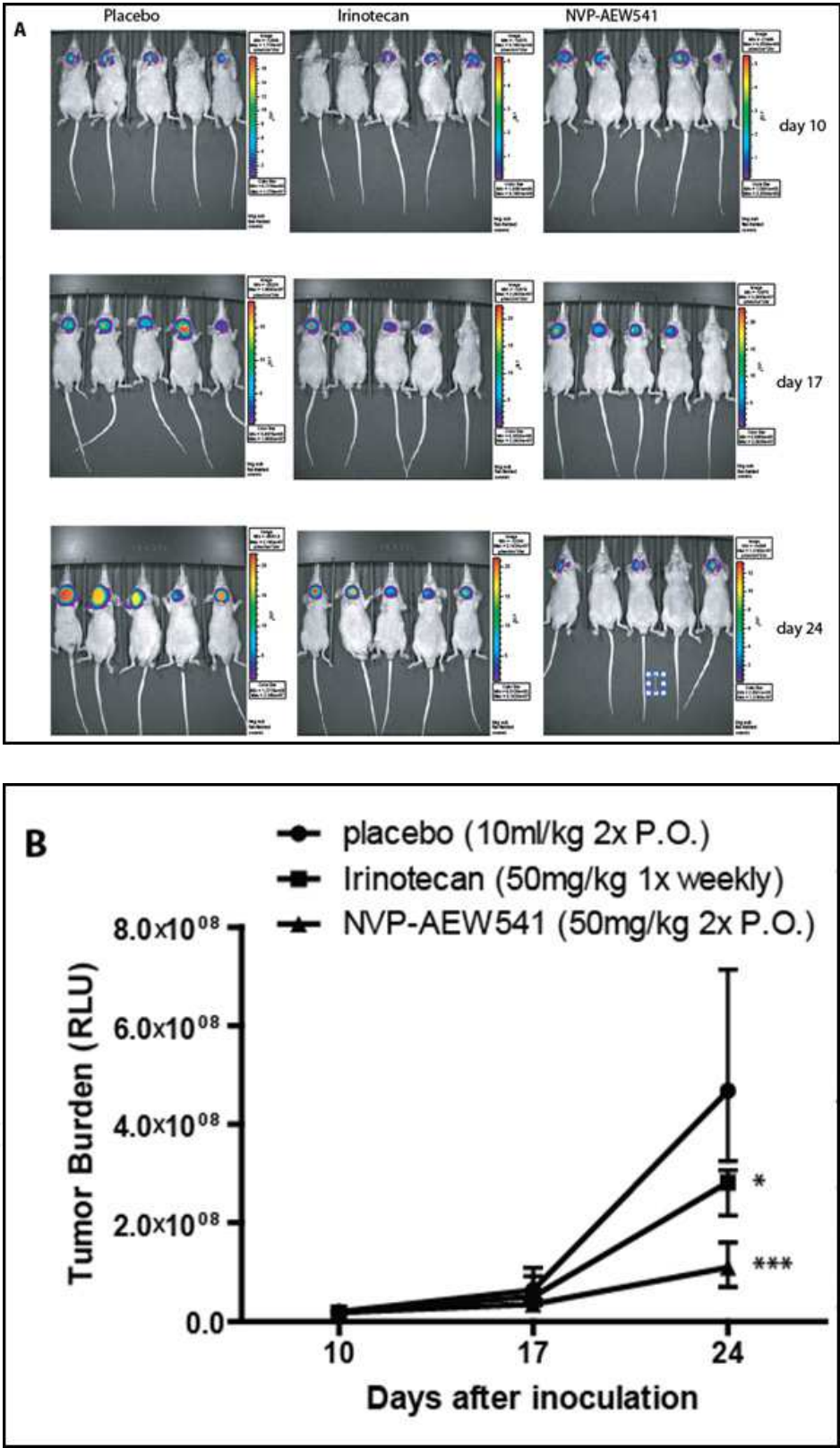
Fig. 11. Realtime bioluminescent imaging (BLI) to monitor the growth of orthotopically inoculated FTC cells treated with placebo, Irinotecan or NVP-AEW541. A) Representative images of mouse at days 10, 17, and 24 after inoculation. (B) Quantification of biochemically

measured luciferase activity (RLU), an indicator of tumor burden to assess the effect of drugs on the respective days shown in panel (A). Tumor growth was significantly inhibited in the NVP-AEW541 treated group as compared to the placebo treated group. Median and range is shown for each point on the graph. Statistics were performed by Student's t test, and significance (P < 0.05) relative to control is noted in the graph.

tumors obstructing the esophagus also partially affected the outcome of survival in these mice. Despite the difficulties, NVP-AEW541 significantly inhibited the growth of orthotopic Wro tumors in nude mice (Fig. 11A) as compared to control or Irinotecan-treated mice (Fig. 11B). Furthermore, microvessel density (MVD) was also significantly decreased after treatment with this compound (Fig. 12) and correlated with decreased VEGF secretion by vascular endothelial growth factors (VEGF) WRO cells treated with NVP-AEW541 in an in vitro ELISA assay (see below). These experiments were conducted only once. Pictorial data has been presented from a representative group of mice and quantitative analysis is based on data obtained from 6 – 8 mice/group.

5.5 Both IGF-IR antibody A12 and SMTKI inhibitor NVP-AEW541 inhibit IGF-I signaling and tumor angiogenesis in orthotopic ATC and FTC models

IGFs have been found to promote the growth, survival, and migration of tumor cells, as well as to induce the syntheses of vascular endothelial growth factors A and C and matrix metalloproteinase 2, which may favor the development of the blood supply essential for the progressive growth of primary malignancies and the development of metastases[71-73]. A recent study was conducted to determine whether anti IGF-IR therapy inhibits IGF-I signaling and tumor angiogenesis *in vivo*. Immunohistochemical analyses of ATC tumors treated with A12 or A12 and Irinotecan showed a decrease in pIGF-IRβ, pAkt, and PCNA staining and an increase in apoptosis in both the treatment groups *in vivo*[2]. These observations were in striking contrast to A12's effect *in vitro*, where treatment of ATC cell lines with A12 failed to induce an apoptotic response. A possible explanation for this discordance could be that A12's additional ability to induce antibody-dependent cell cytotoxicity (ADCC) and complement-mediated cell death (CDC) *in vivo* results in the activation of immune response and inhibition of tumor growth. Strangely enough, despite A12's ability to induce apoptosis in vivo, the survival rate of mice treated with A12 alone was not improved over that of the placebo-treated mice (p=0.578). Contrarily, mice in the combination treatment group showed significant survival advantage (p=0.002) over the mice in control group[2]. These observations suggest that anti-IGF-IR therapy when given in combination with other therapeutic strategies augments anti-tumor effects. To further determine if anti-IGF-IR therapies can be used to inhibit thyroid tumor angiogenesis, we sought to determine whether IGF-IR regulates any molecular targets of angiogenesis as has been reported for colorectal cancer cells[74]. In these studies, stimulation of IGF-IR in colorectal cells has been shown to induce the expression of VEGF to regulate development of new blood vessels[75]. Furthermore, blockade of IGF-IR led to significant down-regulation of VEGF and inhibition of tumor growth and lymph node metastasis of these tumors[76]. To test whether over-expression of IGF-IR in follicular carcinoma cells (WRO) increases VEGF secretion as reported for the colon carcinoma cells, 5×10^5 vector transfected (WRO-puro) or wild type IGF-IR over-expressing (WRO-wt) or constitutively active IGF-IR expressing (CA-IGF-IR) WRO cells were allowed to attach and grow for
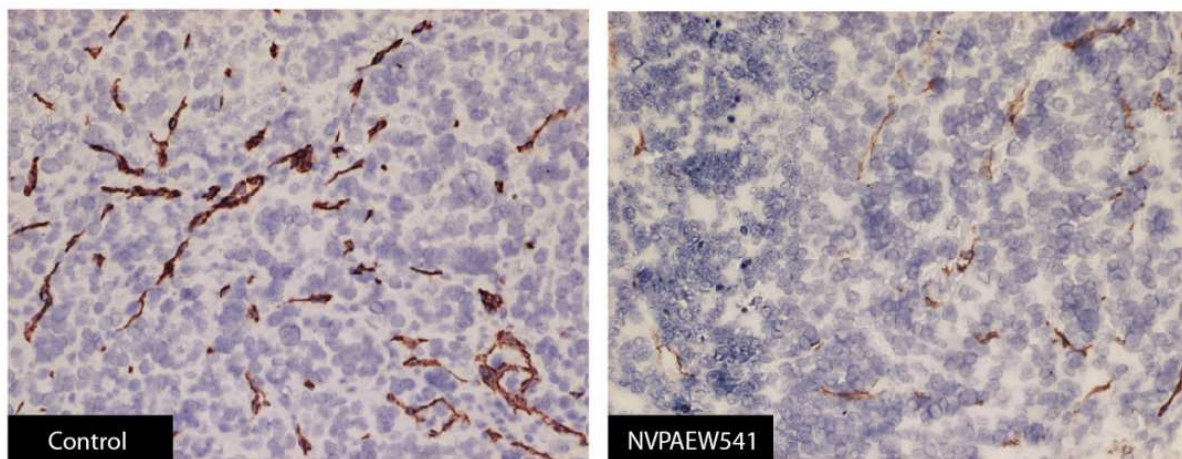
Fig. 12. Histological analysis of tumor sections from Wro-Cd8IGF-IR orthotopic tumors untreated or treated with NVP-AEW541. Tumor samples from representative animals were resected, fixed, and processed for immunohistochemistry with anti CD31 antibody. NVP-AEW541-treated samples showed noticeable reduction in angiogenesis compared to control. Magnification 200X.

24hrs. The supernatant was then replaced with 2 - 3 mL fresh 2% heat-inactivated fetal bovine serum containing culture medium and further cultured for 24 hours. The concentration of VEGF165 in each supernatant was determined using commercially available enzyme-linked immune-absorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN). Culture supernatant from MDA1986 cells was used as positive control for VEGF-A secretion. Both the IGF-IR overexpressing WRO cells and the CA-IGF-IR cells secreted significantly higher levels of VEGF-A as compared to the vector control cells (WRO Puro). We further observed that the treatment of these cells with the SMTKI NVP-AEW541 partially suppressed IGF-IR induced up-regulation of VEGF secretion (Fig. 13; $P < 0.005$ in both cases), suggesting that VEGF secretion in part may be regulated through the IGF-IR signaling pathway.

However, even though NVP-AEW541 is highly sensitive to IGF-IR, it does demonstrate inhibitory activity towards IR, MAPK and PI3K. Thus involvement of other TKIs in the process cannot be overruled. Nonetheless these data does provide credence to our hypothesis that thyroid tumor angiogenesis may be the result of enhanced VEGF secretion in IGF-IR over-expressing tumors. Additional evidence for involvement of IGF-IR in thyroid tumor angiogenesis came from our observations in A12 treated orthotopic ATC specimens. Staining of these tumors for CD31 showed a significant decrease in microvessel density especially in tumors treated with A12 alone and in tumors treated in combination with Irinotecan. Additional staining for pIGF-IR in the tumor endothelium of these tumors confirmed that this response was due to the loss of phosphorylation of IGF-IR in tumor-associated endothelium of A12-treated tumors[2]. In summary, these studies suggest that thyroid tumor angiogenesis is partially regulated through IGF-IR signaling in both ATCs and FTC orthotopic xenografts. To further confirm if human thyroid specimens showed a correlation between IGF-IR signaling and local metastasis, we measured the pIGF-IR content of human thyroid cancer specimens with and without lymph node metastasis. We observed that pIGF-IR expression when computed as an index (pIGF-IR index) varied amongst

thyroid tumors. Particularly, pIGF-IR index of thyroid tumors with lymph node metastasis was lower than for ones without lymph node metastasis, suggesting a direct role for IGF-I signaling in local thyroid tumor metastasis.
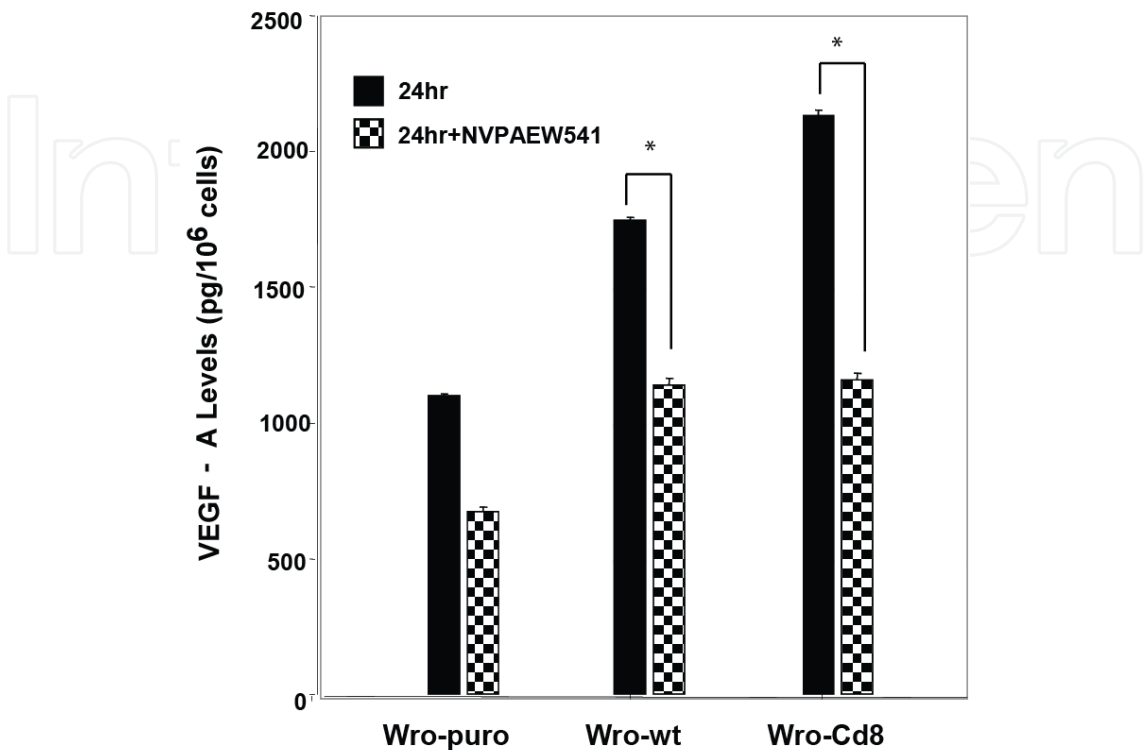

Fig. 13. Effect of NVP-AEW541 on VEGF - A secretion by follicular thyroid carcinoma cells that over express either the wt-IGF-IR or the constitutively activated IGF-IR or are vector transfected (Wro-puro). 5×10^5 cells/ml were allowed to attach and grow for 24hrs in 6 well dishes. The supernatant was then replaced with 2 mL fresh culture medium containing 2% heat-inactivated fetal bovine serum and further cultured for 24 hours in the presence of IGF-IR inhibitor NVP-AEW541. The dose of NVP-AEW541 was predetermined using a standard MTT assay and was found to bring about 50% inhibition in cell growth at a concentration of 0.2uM. VEGF secretion was evaluated by ELISA in conditioned medium of the wro clones 24 hrs after exposing to the drug using commercially available enzyme-linked immuno-absorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN). Results are presented in pg VEGF165 /10^6 cells/24h for control and treated samples as mean ± STE from three separate experiments. MDA1986 cells were used as positive control for VEGF-A secretion. VEGF165 was undetectable in media without the cells (data for controls not shown)

6. Conclusions

IGF-I/IGF-IR axis plays a pivotal role in thyroid tumor progression, particularly by enhancing the angiogenic response of these tumors. Thus targeting IGF-1R signaling particularly in FTC, PTC and more differentiated ATCs could have significant therapeutic potential. Many compounds that directly target IGF-1R have now been developed and the two most investigated strategies to date have used IGF-1R tyrosine kinase inhibitors such as NVP-AEW541 and anti-IGF-1R monoclonal antibodies such as A12. We tested the specificity

of both these compounds in orthotopic models of ATC and FTC and have presented data that shows their efficacy. Furthermore, blockade of IGF-IR suppressed IGF-I signaling, induced apoptosis in vitro and in vivo. Both compounds also suppressed angiogenesis although via different mechanisms. Combining A12 treatment with standard of care chemotherapeutic drug Irinotecan enhanced the cytotoxic effects of the chemotherapeutic drug. Our results are in agreement with previously reported data in other solid tumors and suggest that blocking IGF-IR with A12 or NVP-AEW541 seems to be a potential avenue for treating thyroid cancer through their direct antitumor effects and their effects on tumor vasculature. Additionally, we reviewed additional strategies that are under clinical development or in clinical trial for targeting this axis in cancer. Nevertheless, as clinical developmental programs progress careful attention must be paid to the potential side effects of this approach, especially since IGF-I signaling plays an equally important role in cell growth, energy metabolism and differentiation. A closer look on the effect of dose and schedule on toxicity are also warranted.

7. Acknowledgements

8. References

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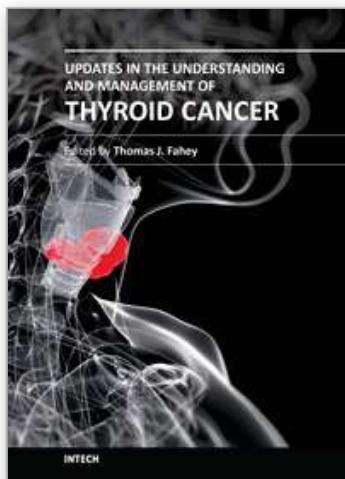
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