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NOVEL TREATMENTS FOR ADVANCED THYROID CANCER AND ELUCIDATION OF BIOMARKERS

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NOVEL TREATMENTS FOR ADVANCED THYROID CANCER AND ELUCIDATION OF BIOMARKERS

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CONTENTS

1

GENERAL INTRODUCTION AND OUTLINE OF THIS THESIS

INTRODUCTION

Thyroid cancer is the most prevalent endocrine malignancy, estimated to account for 96% of cancers of the endocrine system and 66% of deaths due to cancers of the endocrine system in 2012.[1] The incidence of thyroid cancer throughout the world seems to be increasing. In part the increased incidence might be explained by more frequent and improved diagnostic imaging, thereby identifying so called "incidentalomas". However, the number of patients that die due to this disease also increases. The current incidence in Europe is 49/1.000.000 per year, with a nearly 3 times higher incidence in women.[2]

Thyroid cancer is a heterogeneous disease which is classified into differentiated forms of thyroid carcinoma (DTC), undifferentiated (anaplastic) thyroid carcinoma (ATC) and medullary thyroid carcinoma (MTC). DTC and ATC together are classified as non-medullary thyroid cancer (NMTC, Table 1). Differentiated thyroid carcinoma is by far the most common type (95%), and includes papillary (PTC) (80%) and follicular (FTC) subtypes (10-15%). Furthermore oncocytic or Hurthle cell metaplasia can occur in PTC and FTC due to accumulation of mitochondria in the tumor cells. The latter is recognised as pink cytoplasmic colouring in routine hematoxyline and eosin tissue staining. To complicate the distinction of NMTC also follicular variants of PTC (FVPTC) are recognized. The majority of DTCs are slowly progressive, and, when identified at an early stage, most frequently cured with adequate surgical management and post-operative radioactive iodine 131-I ablation therapy (RAI). Metastatic DTC that has become inoperable or refractory to radioactive iodine therapy however, is associated with a poor survival (Table 1). NMTC cancers that are refractory to RAI therapy mostly comprise a subset of PTCs with activating *BRAF* gene variants and oncocytic variants of FTC (FTC-OV).[3] Remarkably FTC-OV is very low frequent in consecutive series of NMTC. MTCs and especially ATCs metastasize in up to 50% of patients, thus giving an even worse prognosis.[4] Furthermore ATC present itself as a rapidly
expanding cervical mass, that is often irresectable and leads to death within three months after diagnosis. Results of conventional treatment modalities (radiotherapy and/or chemotherapy) are disappointing, although there are exceptions. Therefore, new therapies are needed. As a result of the increasing knowledge of the biologic basis for thyroid cancer development, therapeutic agents that target involved biologic abnormalities have been identified. Based on these insights multiple clinical trials have been performed in the past decade. In this introduction we describe new treatment modalities in RAI refractory NMTC and MTC.

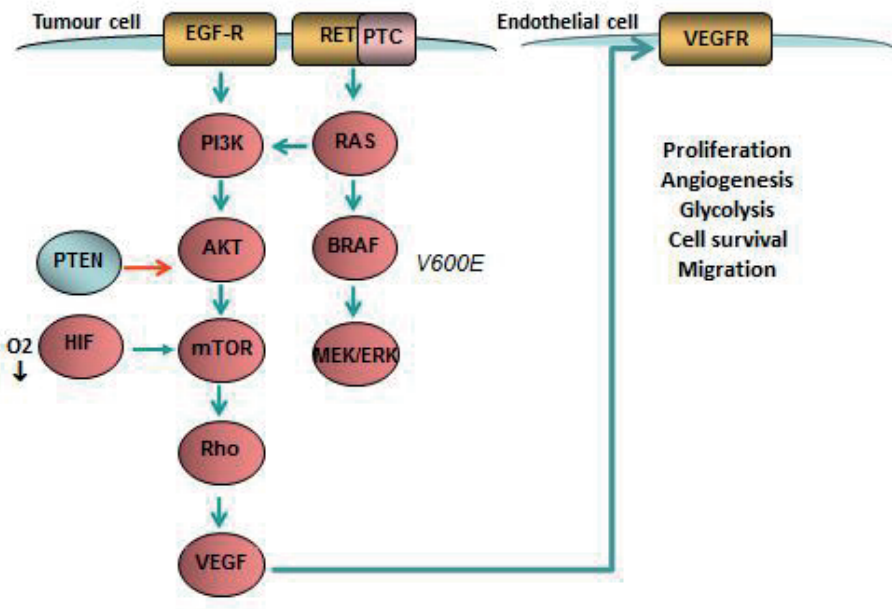
Table1: Thyroid cancer: tumor type, age, prevalence and survival

Tumor type		Age (Y)	Prevalence	10-year survival	
				Nonmetastatic disease	Metastatic disease
Differentiated	Papillar Follicular	10-60 25-70	60-70% 20-30%	90-95%	5-10%
Medullary		10-60	5%	75%	10%
Anaplastic		>60	5-10%	<5%	0

MOLECULAR PATHOGENESIS OF NMTC

For an overview of the signaling pathways involved in thyroid cancer and described below, see Figure 1.

Figure 1 Signaling pathways involved in thyroid tumorigenesis



PTC

Genetically classic PTC is characterized by either activating *BRAF* variants (mostly V600E) or *rearranged during transfection* (*RET*) proto-oncogene rearrangements leading to the fusion of the *RET* tyrosine kinase domain to the 5′ end of a variety other genes that are constitutively active in follicular thyroid cells. This results in the generation

of a variety of chimeric oncogenes and proteins denoted RET/PTC whose expression is under the control of promoters provided by the fused genes, and thus leading to ligand-independent activation of RET in papillary thyroid cancer.[5] To date, 12 of these chimeric RET/PTC proteins have been described. These *RET/PTC* gene fusions occur in 16 to 25% of PTC cases. Normal functioning BRAF is a putative downstream signal transducer for the RET/PTC fusion products. *RET/PTC* gene fusions are often found in pediatric PTC, either being unexplained or being the result from nuclear plant accidents as seen in Tsernobyl or recently in Fukushima. PTC with gene fusions at later ages can alternatively be the late result of medically administered radiation therapy. Gene fusions involving the *neurotrophic tyrosine kinase receptor* (*NTRK*) genes lead to transcription of chimeric Trk proteins, resulting in subsequent activation of intracellular signaling molecules, including RAS, PI3K, and MAPK, thereby stimulating cellular proliferation, differentiation, and survival. *NTRK* gene rearrangements are found in 1-5% of papillary thyroid carcinomas, especially in patients with a history of radiation exposure.[6-10] However especially at early ages PTCs are mostly sensitive to the given RAI therapies, thereby not posing a problem of disease recurrence.

In benign thyroid cells BRAF is an important regulator of thyroid-specific protein expression and proliferation.[11] Constitutively activated BRAF through mutation is responsible for the development of papillary thyroid carcinoma (PTC), that can potentially progress towards anaplastic carcinoma. Kebebew et al. reported significant associations of *BRAF* V600E with recurrent and persistent disease with a higher rate of lymph node metastasis and higher TNM stage in PTC and [12]. BRAF was therefore initially considered to be an attractive target for therapy in PTC and ATC with activating V600E *BRAF* variants.[13]

The role of the *anaplastic lymphoma kinase* (*ALK)* gene rearrangements as oncogenic drivers has been well established in preclinical models including transgenic mouse models [14,15]. *ALK* is a receptor tyrosine kinase (RTK) that activates the MAPK/ERK and PI3K/AKT pathways, promoting cell proliferation and survival. Various *ALK* fusions (*EML4-ALK, GFPT1-ALK, TFG-ALK,* and *STRN-ALK*) are reported in thyroid cancer patient tumors [16,17].

FVPTC

An option is to classify FVPTC only when there is a complete absence of "papillae" and only the nuclear features of PTC are seen (orphan Annie's eyes, and nuclear indentation). When being strict about this morphological distinction no *BRAF* V600E variants or *RET/PTC* gene fusions are found in FVPTC. More often *RAS* (*HRAS, NRAS, KRAS*) gene variants or *Paired Box* 8 /*peroxisome proliferator-activated receptor gamma* (PAX8/PPARɣ) gene rearrangements are identified. These molecular alterations are also encountered in FTC (see below).

FTC

FTC classically shows *RAS* (*NRAS* or *HRAS*) or chromosomal translocation leading to a PAX8/PPARγ gene rearrangement. The transcription factor PAX8 plays a role in the expression of multiple thyroid specific genes. The *PAX8/PPARγ* gene rearrangement is found in 30-40% of follicular thyroid carcinomas. This rearrangement exerts a negative effect on the tumor suppressor *PPARγ* and activates genes responsive to PAX8.[18-22]

FTC-OV

In FTC-OV gene variants or fusions in cancer driver genes are relatively rare although those found (e.g. *FLCN*, *MEN1*, *mTOR, PTEN, PIK3CA*, *TP53*, and *TSC2*) are frequently involved in metabolic switches. Possibly additional tumor drivers need to be identified. Using a new method for DNA content analysis in combination with single nucleotide polymorphism (SNP) technology it was demonstrated that many FTC-OV actually show a near-homozygous genome (NHG) in which a phase of near-haploidization is followed by endoreduplication or genome doubling of the entire NHG[23-26]. The observations regarding a NHG have been confirmed by others [27,28]

ADDITIONAL GENE ALTERATIONS IDENTIFIED IN NMTC CANCERS

In comparison with other types of non-thyroid cancers in differentiated PTC (and FTC) only few additional gene variations can be found. These comprise either variants in *PIK3CA* and *PTEN* or *human (h)-TERT* gene promoter variants. *TERT* promoter mutations are seen in ATC (45-73%), PDTC (40%), FTC (14%) and PTC (9%) and can occur concomitantly with MAPK-pathway mutations.[29-32] *TERT* mutations are associated with aggressive clinicopathological behavior and a high risk of recurrent disease, particularly when accompanied by a *BRAF* mutation.[33]

It has been suggested that alterations in DNA repair mechanism are involved in progression to aggressive forms of PTC.[34]. For instance *CHEK2* and *PPM1D* gene variations can occur. *CHEK2* is a tumor suppressor gene involved in preserving genome stability by repairing DNA double strand breaks. PPM1D is a phosphatase that inhibits p53-mediated transcription and apoptosis.[35,36]

MOLECULAR PATHOGENESIS OF MTC

Activating *RET* mutations are present in more than 95% of hereditary MTC cases and in 20 to 50% of sporadic MTC cases. Several mutational hotspots of the RET gene have been described to be tumorigenic. Furthermore, a common specific activating point mutation, M918T, appears to be a strong negative prognostic indicator for metastasis-free survival and survival (OS). Ten-year survival was approximately 45% in subjects with a confirmed M918T mutation, while it is reported to be as high as 90% in absence of the mutation.[37] In up to 68% of patients without a *RET* mutation, *RAS* mutations have been found with *HRAS* and *KRAS* variants being most frequently observed.[38-41] In addition to *RET* and *RAS*, the *vascular endothelial growth factor receptor* (*VEGFR*) and *MET* proto-oncogenes may be implicated in the pathogenesis of MTC. Transduction of thyroid cells with mutant RET results in upregulation of MET.[42] Finally *mTOR*, an effector of the PI3K/AKT pathway, plays an important role in the development of MTCs. [43]

MOLECULAR PATHOGENESIS OF POORLY DIFFERENTIATED AND ATC

ATC either derives from PTC with V600E *BRAF* mutations or from FTC-OV. In resected ATC specimen often only after extensive sampling these more differentiated foci with either PTC or FTC are encountered. Additional *TP53* gene variants are then driving the aggressive ATC tumor fractions. In 9% of poorly differentiated, 4% of anaplastic and 1 % of papillary thyroid cancers fusion of the *striatin* (*STRN*) gene and *ALK* gene have been reported.[44]

RAS/RAF/MAPK PATHWAY IN THYROID CANCER

In a review of 11 studies in thyroid cancer, it was noted that up to 50% of follicular and 12% of FTC-OV cell malignancies contained *RAS* mutations.[45] The RAF proteins are cytoplasmic serine/threonine protein kinases that are downstream effector molecules of RAS. Of these, BRAF is the most efficient at phosphorylating mitogen activated protein kinase (MAPK) and is important in proliferative as well as apoptotic pathways. [46] Point mutations leading to BRAF signaling independent of binding to RAS as seen in PTC underlines the significance of the RAS/RAF/MAPK pathway in thyroid cancer.[47] The PI3K/AKT pathway on the other hand plays an important role in cell proliferation and survival and has been found by others to be aberrantly activated in thyroid tumors. [48-51] An important player in this pathway is the PI3KCA subunit that in turn is also regulated by RAS. In a study by Hou et al., a progressive activation of the PI3K/AKT

pathway and associated methylation of *PTEN*, known to suppress this pathway, was found in thyroid adenomas, follicular and anaplastic thyroid cancers.[52]

ANGIOGENESIS IN THYROID CANCER

Concomitantly with MAPK pathway mutations in human thyroid cancer, *BRAF* V600E is associated with VEGF overexpression, which in turn is associated with increasing tumor stage and invasiveness.[53] In 1997, Soh et al. observed extensive angiogenesis in thyroid cancer cells that had been xenografted into mice. By using cell lines from human differentiated thyroid cancers (including FTC-OV) and MTC they noted a higher expression of *vascular endothelial growth factor* (*VEGF*) mRNA and protein in thyroid cancer in comparison to normal thyroid tissue.[54]

NEW TREATMENT MODALITIES IN THYROID CARCINOMA

As a result of the increasing knowledge of the biologic basis for thyroid cancer development, therapeutic agents that target these biological abnormalities have been identified. Multiple clinical trials have been performed in the past decade (table 2).

I. TYROSINE KINASE INHIBITORS

Monotarget kinase inhibitors

Gefitinib (ZD1839) is an oral Epidermal Growth Factor Receptor (EGFR) TKI. The EGFR is highly expressed in malignant thyroid tissue and mutations of the EGFR gene have been described in thyroid cancer. Moreover, EGFR contributes to RET activation, signaling and growth stimulation and is associated with poor prognosis in DTC.[55] Pennell et al. studied the effectiveness of gefitinib in a phase II trial with a mixed cohort of thyroid cancer patients. Four percent of patients showed disease reduction. However, this was not qualified as partial response. Overall, 24% of patients achieved stable disease lasting at least 24 weeks. Median progression free survival was almost 16 weeks, with the exception of MTC patients, in which the median PFS was less than 12 weeks.[56] Gefitinib does not have a registration for disseminated thyroid cancer.

AZD6244 is a potent, selective, non-competitive inhibitor of MEK1/2 that has been studied in a phase I study and has shown interesting activity in 2 advanced thyroid cancer patients with stable disease for at least 5 months.[57] A phase II trial conducted in advanced DTC patients showed a partial response in 3% of patients, 66% of patients had stable disease and the median PFS was 32 weeks.[58]

Multikinase inhibitors

Axitinib (AG-013736) is an oral TKI that effectively blocks all of the VEGFRs. One of 5 patients with thyroid carcinoma included in a phase I trial experienced tumor shrinkage, which however, was not qualified as a partial response.[59] A phase II trial by Cohen et al. studied the efficacy of axitinib in advanced or metastatic thyroid carcinoma of any histology (30 PTC, 15 FTC and 11 MTC). A partial response was seen in 31% of the DTC patients and 18% of the MTC patients. Stable disease lasting more than 16 weeks was reported in 38% and the median PFS was 18.1 months.[60] Another phase II study in 52 DTC patients reported a PR and SD >16 weeks in 18 (35%) patients. The median PFS was 16 months, the median OS 27 months.[61]. However, axitinib has not been registered for thyroid cancer.

Motesanib (AMG 706) is an oral TKI targeting the VEGFR 1-3, RET and c-KIT. In a phase I trial by Rosen et al. a 50% overall response rate was observed in patients with advanced thyroid carcinoma.[62] Based on these results a multicenter phase II trial was initiated, testing the efficacy of motesanib therapy in patients with progressive DTC and progressive or symptomatic MTC. In 14% of the DTC patients partial response was confirmed, and another 35% of these previously progressive patients maintained stable disease for at least 24 weeks. The median response duration was 40 weeks. [63] In a phase II trial in MTC patients by Schlumberger et al. only 2% of patients had confirmed partial response, but stable disease for at least 24 weeks was reported in 48% of patients. Median PFS was 48 weeks.[64]

Vandetanib (ZD 6474) is an oral TKI that targets VEGFR 2 and 3, RET and EGFR. Vandetanib effectively inhibits RET/PTC3 chromosomal translocations found in some PTC and M918T RET mutations occurring in MEN2B-associated and some sporadic MTC.[65] In a phase II trial, Wells et al. studied the efficacy of vandetanib in patients with metastatic hereditary forms of MTC. Confirmed partial response was reported in 17% of patients, where another 53% had stable disease lasting at least 24 weeks.[66] A second phase II trial in advanced hereditary MTC by Robinson et al. showed a partial response rate of 16% and stable disease 24 weeks or longer in 53% of patients.[67] Results of a randomized placebo controlled multicenter phase III trial in 331 patients with locally advanced or metastatic MTC showed an objective response in 43% of patients, all which were durable. Median PFS was 30 months in the vandetanib group and 19 months in the placebo group ($p<0.001$). The OS did not significantly differ between the two groups.[68] Vandetanib has a registration for metastatic medullary thyroid carcinoma.

Sorafenib (BAY 43-9006) is an orally active TKI targeting BRAF, VEGFR 1 and 2 and RET, conducting pro-apoptotic and anti-angiogenic actions. Several phase II studies with Sorafenib in patient with advanced DTC have been conducted, showed promising results.[69-72] In the Phase III DECISION trial, investigating the efficacy and safety of sorafenib in patients with advanced, RAI-refractory DTC, 417 patients were randomised between sorafenib twice daily 200 mg and placebo with the option of crossover in

case of disease progression. The median progression-free survival in the placebo and sorafenib group was 5.8 months and 10.8 months respectively (HR 0.587; p < 0.0001). [73] In a small pilot study in patients with metastatic MTC, responses were described in 40% of the patients.[74] Lam et al performed a phase II trial in which the efficacy of sorafenib in hereditary (arm A) and sporadic (arm B) metastatic MTC patients was examined. They included 16 patients with sporadic MTC. One patient had a PR (6.3%) and 14 patients had stable disease (87.5%). Median PFS was 17.9 months. Arm A was prematurely terminated because of slow accrual.[75] Sorafenib has a registration for DTC.

Sunitinib (SU11248) is an oral TKI of VEGFR 1-3, RET, and RET/PTC subtypes 1 and 3. Results of a phase II trial in patients with MTC (n=25) showed a PR in 8 (32%) and SD in 13 (52%) patients.[76] Another phase II trial in 28 DTC and 7 MTC patients reported a CR in 3%, a PR in 28% and SD in 46% of all patients combined. Median PFS was 12.8 months.[77] Recent data of a phase II trial in 11 patients with DTC demonstrated a CR in 9%, a PR in 18% and SD in 45% of patients with the median PFS being 11.2 months.[78]. Sunitinib does not have a registration for thyroid cancer.

Imatinib (STI571) is an oral TKI of BCR-ABL and c-KIT. Its function is based on its inhibition of RET autophosphorylation and RET mediated cell growth. So far, two small phase II trials that studied the efficacy of imatinib in patients with metastatic MTC have been completed. In both trials no response was confirmed and only a few patients achieved stable disease.[79,80] Furthermore, a phase I trial with MTC patients treated with imatinib combined with dacarbazine and capecitabine did not report objective responses.[81]

Pazopanib (GW786034) is an orally bioavailable TKI that targets VEGFR 1-3 and c-KIT. Its antitumor activity in advanced and progressive DTC was demonstrated in a phase II trial with 39 patients. Partial responses were confirmed in 49% of patients. Median PFS was 12 months.[82]. Pazopanib has not been registered for thyroid cancer.

Cabozantinib (XL184) is an oral inhibitor of RET, c-MET and VEGFR 1 and 2. C-MET activation triggers tumor growth and angiogenesis. Moreover, in patients with PTC and MTC overexpression and frequent mutations of the c-MET receptor have been reported.[42] A phase I trial examined the efficacy of cabozantinib in patients with advanced malignancies, including patients with MTC. In MTC patients with measurable disease, 29% had a confirmed PR and 68% had either confirmed PR or prolonged stable disease ≥ 6 months.[83] A randomized placebo controlled phase III trial (EXAM) was conducted in patients with advanced or metastasized MTC. Median PFS was 11 months in the cabozantinib group and 4 months in the placebo group (p<0.001) with an overall RR of 28% and OS HR of 0.83. Remarkable was the significant longer PFS in patients harboring a RET mutation (60 vs. 20 weeks).[84] Cabozantinib is now registered for the treatment of advanced MTC. The possible role of cabozantinib in patients with DTC is currently being investigated in a phase II clinical trial (NCT02041260). Cabozantinib has

a registration for locally advanced and metastatic medullary thyroid carcinoma.

Vemurafenib (PLX 4032) is an orally administered small molecule that specifically inhibits only the V600E mutant BRAF kinase, without appreciable impairment of wild-type BRAF protein or other RAF kinases. Three patients with PTC and documented *V600E BRAF* mutations have been treated in a phase I study, with 1 of the 3 experiencing a partial response and the other 2 having prolonged stable disease.[85] Data from a phase II clinical trial in patients with PTC showed a PR in 38% of patients not previously treated with a TKI and in 26% of patients previously treated with a TKI. The median PFS was 16 and 7 months respectively.[86]. Vemurafenib does not have a registration for thyroid cancer.

Lenvatinib (E7080) is also an inhibitor of multiple TKs, especially VEGFR's, c-KIT, PDGFR beta stem cell factor receptor, RET and FGFR1-4.[87-89] Based on the encouraging results of a phase II clinical study with lenvatinib in patients with RAI refractory DTC, the placebo controlled phase III SELECT trial was conducted (lenvatinib n=261, placebo n=131). The RR was 64% (4 CRs and 165 PRs) in the lenvatinib group and 1.5% in the placebo group ($p<0.001$). Median PFS was 18 and 4 months respectively. Median OS was not reached in both groups.[90,91]. Lenvantinib has been registered for locally advanced and metastatic DTC.

Selumetinib is a non-ATP competitive MAPK kinase inhibitor (MEK1/2). A phase 2 study of selumetinib in 39 PTC patients reported a PR in 3% and SD in 54% of the 32 evaluable patients with a median PFS of 8 months in patients harboring a *BRAF* mutation and 3 months in patients without a BRAF mutation.[92] More recent data of a phase II trial in patients with metastasized DTC (n=20) showed a PR and SD in 25% and 15% respectively. Remarkable was the enhanced RAI uptake and tumor reduction in patients with a NRAS mutated tumor.[93] This drug is still under investigation.

Everolimus (RAD001) is an orally available derivative of rapamycin that interferes with the regulation of cell cycling, cell growth and cell survival mechanisms through binding to the mammalian target of rapamycin.[43] A phase II study of everolimus in patients with advanced thyroid cancer of all histologic subtypes (n = 38), reported a partial response (PR) and durable stable disease (SD) in 5% and 45% of patients, respectively. The median PFS (mPFS) was 47 weeks.[94] Another study of everolimus in DTC (n = 31) showed 1 (3%) patient with a PR and 18 (58%) with durable SD. PFS was 16 months, and 1-year survival was 76%.[95] Recently published results of a phase II trial in patients with advanced DTC reported SD in 17 (65%) patients, of which 15 (58%) showed SD >24 weeks. Median PFS and OS were 9 and 18 months, respectively.[96] Wagle *et al.* showed 1 patient diagnosed with ATC derived from FTC-OV with striking response upon giving everolimus due to a homozygous somatic *TSC1* variant. This patient showed relapse when the tumor was selected for a secondary somatic *MTOR* variant.[97] Everolimus does not have a registration for thyroid cancer.

Crizotinib is an inhibitor of ALK, MET and ROS1 and is approved for the

treatment of patients with ALK- or ROS-positive advanced non-small-cell lung cancer (NSCLC). A study with crizotinib in patient with ALK-positive malignancies (excluding NSCLC) showed a PR lasting 14 weeks in a patient with advanced MTC.[98] Godbert *et al.* reported an exceptional response in a patient with ALK-rearranged ATC and lung metastases. Over 90% of all pulmonary lesions responded to crizotinib treatment and this response was still ongoing after 6 months.[99]

IMMUNOTHERAPY

Despite the low mutational load of most thyroid cancers, which would theoretically indicate a lack of potential benefit upon giving immunotherapy, targets were investigated. Several reports demonstrated higher programmed death-ligand 1(PD-L1) expression in thyroid tumors. Furthermore, PD-L1 levels were higher in advanced tumors and more frequently expressed in ATC, suggesting that PD-L1 expression is a late event in thyroid carcinogenesis.[100-102] In addition, it was demonstrated that *BRAF* V600E mutated PTCs frequently express PD-L1. These findings show that *BRAF* V600E can induce promotion of tumor immune escape mechanisms, may contribute to the aggressive tumor behavior of *BRAF* V600E mutated tumors.[103]

Preliminary results of a study with pembrolizumab in patients with advanced PTC or FTC, who failed standard treatment and showed PD-L1 expression, reported a PR in 2 out of 22 patients (9%). SD was seen in 55% of patients, PFS and OS at 6 months were 59% and 100% respectively.[104] Kollipara *et al*. reported an exceptional response in 1 patient with a *BRAF* and PD-L1 positive recurrent ATC, treated with vemurafenib and nivolumab. After 20 months the patient continues to be in complete radiographic and clinical remission.[105] Multiple clinical trials evaluating immune checkpoint inhibitors in patients with thyroid cancer are ongoing (https://clinicaltrials.gov).

Table 2: Summary of studies in thyroid cancer

Drug	Tumor type	N	RR	SD	PFS	Reference
Monotarget kinase inhibitors						
Gefitinib	DTC	18	0#	24 % (>24 wks)#	16 wks #	Pennell et al. 2008
	MTC	4			<12 wks	
	ATC	5			16 wks #	
AZD6244	DTC	39	3%	66%	32 wks	Lucas et al. 2010
	MTC	2	-	100% (>20 wks)	-	Banerji et al. 2010
Multikinase inhibitors						
Axitinib	All types	5	0%	-	-	Rugo et al. 2005
	DTC	45	31%	42%	18mnths#	Cohen et al. 2008
	MTC	11	18%	27%		
	DTC	52	35%	35%	27 mnths	Locati et al. 2014
Motesanib	DTC+MTC	71	7%	49% (>12 wks)	-	Rosen et al. 2007
	DTC	93	14%	35% (>24 wks)	40 wks	Sherman et al. 2008
	MTC	91	2%	28% (>24 wks)	48 wks	Schlumberger et al. 2009
Vandetanib	DTC	145	8%	57%	11 mnths	Leboulleux et al. 2012
	MTC	30	20%	53%	28 mnths	Wells et al. 2010
	MTC	19	16%	53%	6 mnths	Robinson et al. 2010
	MTC	231	45%	42%	-	Wells et al. 2012
	MTC	60	20%	55%	16 mnths	Chougnet et al. 2015
Sorafenib	DTC	41	15%	56% (≥24 wks)	15 mnths	Kloos et al. 2009
		30	23%		79 wks	Gupta et al. 2008
		26	27%		18 mnths	Schneider et al. 2012
		207	12%	42% (≥26 wks)	11 mnths	Brose et al. 2013
	MTC	16	6%	88%	18 mnths	Lam et al. 2010
Sunitinib	All types	17	6%	80%	-	Ravaud et al. 2009
	DTC+MTC	43	13%	68%	-	Cohen et al. 2009
	DTC+MTC	35	31%	46%	13 mnths	Carr et al. 2010
	MTC	35	35%	57%	7 mnths	De Souza et al. 2010
Imatinib	DTC+MTC	-	-	-	-	-
Pazopanib	DTC	37	32%	65%	12 mnths	Bible et al. 2009
Cabozantinib	MTC	33	28%	-	11 mnths	Kurzrock et al. 2011
Vemurafenib	DTC	3	33%	66%	12 mnths	Kim et al. 2013
	DTC	51	38%$^{+}$	-	16 mnths^{+}	Brose et al. 2013
			26%$^{+}$		7 mnths^{+}	
Lenvatinib	DTC	58	50%	28%	13 mnths	Sherman et al. 2011
	DTC	261	65%	-	18 mnths	Schlumberger et al. 2015
Selumetinib	DTC	32	3%	54%	8 mnths	Hayes et al. 2012
	DTC	20	25%	15%	-	Ho et al. 2013
Everolimus	All types	38	5%	45%	12 mnths	Lim et al. 2013
	MTC	7	-	57%	8 mnths	Schneider et al. 2015
	DTC	28	-	65%	9 mnths	Schneider et al. 2017

overall outcomes, RR response rate, SD stable disease, PFS progression free survival, DTC differentiated thyroid cancer, MTC medullary thyroid cancer, wks weeks, mnths months, - not reported, $^{+}$ in the TKI naive group and in the group previously treated with a TKI respectively

CONCLUSION

The large development in the treatment of advanced thyroid cancer is due to the unraveling of the carcinogenesis of thyroid cancer. Aberrations in RET/PTC-RAS-RAF-MAPK pathways are present in a high percentage of thyroid cancer, and there are also angiogenesis switch alterations and involvement of other receptor tyrosine kinases, such as EGFR or c-Met. Because of the oncogenic roles of activated BRAF, RET, and RET/PTC kinases, and RET/PTC kinases, the assumption was that specific targeting of these kinases could block tumor growth and induce senescence.[106] As can be shown from multiple clinical trials in thyroid cancer, these assumptions have appeared to be correct. Several promising agents have been found in DTC and MTC (table 2). However, the search for new agents in sequential or combination therapy will still be necessary, since patients eventually become progressive on these agents or do not tolerate them.

OUTLINE OF THIS THESIS

In this thesis, clinical aspects of advanced thyroid cancer were studied. The first part of this thesis is focused on the role of sorafenib in the treatment of patients with advanced thyroid cancer. **Chapter 2** describes the long-term results of a prospective phase II clinical trial to determine the efficacy of sorafenib in patients with advanced radio-iodine refractory differentiated thyroid cancer. Drugs such as sorafenib may induce metaplasia/clonal divergence of metastatic thyroid cancer and cause diagnostic misclassification. Furthermore, Sorafenib is potentially involved in the tumorigenesis of secondary non-cutaneous SCC. In **Chapter 3** we describe three patients with a history of sorafenib treatment for advanced radioactive iodine refractory papillary thyroid cancer who presented with secondary non-cutaneous lesions.

In the second part we report the outcomes of clinical trials with everolimus in patients with advanced thyroid cancer. In **Chapter 4** we report the results of a prospective phase II study on the safety and efficacy of everolimus in patients with advanced MTC. **Chapter 5** evaluates the correlation between everolimus exposure and toxicity and its population pharmacokinetics in patients with advanced thyroid cancer of all histological subtypes. In **Chapter 6** the results of a prospective phase II clinical trial to determine the efficacy and safety of everolimus in patients with advanced follicular-derived thyroid cancer are presented.

This thesis and future prospective are discussed in **Chapter 7**. A summary of this thesis is given in **Chapter 8** in English and in **Chapter 9** in Dutch.

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PART 1
SORAFENIB

2

LONG-TERM ANALYSIS OF THE EFFICACY AND TOLERABILITY OF SORAFENIB IN ADVANCED RADIO-IODINE REFRACTORY DIFFERENTIATED THYROID CARCINOMA: FINAL RESULTS OF A PHASE II TRIAL

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ABSTRACT

Objective

We conducted a prospective phase II clinical trial to determine the efficacy of sorafenib in patients with advanced radio-iodine refractory differentiated thyroid cancer. In this article, the long-term results are presented.

Patients and methods

Thirty-one patients with progressive metastatic or locally advanced radioactive iodine refractory differentiated thyroid cancer received sorafenib 400 mg orally twice daily. The study end points included response rate, progression-free survival (PFS), overall survival (OS), best response by Response Evaluation Criteria in Solid Tumors criteria 1.0, and toxicity.

Results

Median PFS was 18 months (95% confidence interval (95% CI): 7–29 months) and median OS was 34.5 months (95% CI: 19–50 months). Eight patients (31%) achieved a partial response and 11 patients (42%) showed stable disease after a median follow-up of 25 months (range 3.5–39 months). Toxicity mostly included hand foot syndrome, weight loss, diarrhea, and rash.

Conclusion

Sorafenib has clinically relevant antitumor activity in patients with progressive metastatic or locally advanced radio-iodine refractory differentiated thyroid cancer. Sorafenib can nowadays be considered as the standard option in these patients.

Key words

long term outcomes, phase II trial, sorafenib, thyroid cancer

INTRODUCTION

Thyroid cancer is the most prevalent endocrine malignancy, accounting for 95% of cancers of the endocrine system and 66% of endocrine cancer mortality in 2010 [1]. The current incidence in Europe is 49/1 000 000 per year, with a nearly three times higher incidence in women [2].

Differentiated thyroid carcinoma (DTC) is by far the most common (95%) subtype, and includes papillary (PTC, 80%) and follicular (FTC, 10-15%) as well as subtypes like Hurthle cell carcinomas. The majority of DTCs are slowly progressive, and, when identified at an early stage, frequently cured with adequate surgical management and radioactive iodine 131-I ablation (RAI) therapy. However, metastatic DTC that has become inoperable or refractory to RAI therapy is associated with a less favorable prognosis, as 10-year survival varies between 25% to 40%[3,4]. The efficacy of conventional chemotherapy in DTC is negligible, and chemotherapy is therefore no longer recommended in international guidelines [5,6]. As a result of increased understanding of thyroid tumorigenesis, potential targets and novel therapeutic agents that target biological abnormalities have been identified.

In DTC, the role of activated genetic aberrations in the RET-RAS-RAF-MAPK signaling pathway in tumor development and progression has been determined. The B-type Raf kinase (BRAF) V600E mutation has been reported in 29-69% of PTC and is associated with recurrent and persistent disease with a higher rate of lymph node metastasis and higher TNM stage [7,8]. In a review of 11 studies in thyroid cancer, up to 50% of follicular and 12% of Hurthle cell malignancies harbored RAS mutations or RAS-downstream signaling, PIK3CA, mutations [9]. Rearrangements in the RET proto-oncogene occur in up to 25% of PTC, resulting in the generation of chimeric oncogenes responsible for the initiation of tumor formation [10]. Additionally, RAS mutations and RET/PTC translocations also result in aberrant signaling through BRAF. Furthermore, the RET–RAS–RAF pathway leads to vascular endothelial growth factor (VEGF) and VEGF receptor (VEGFR) synthesis through interconnection with the epithelial growth factor receptor (EFGR) activated cascade [11]. In turn, these are the most important regulators in the proces of angiogenesis. Increased vascularity plays a crucial role in tumor growth and generating metastasis [12]. In human thyroid cancer, VEGF over expression is associated with increased tumor stage and invasiveness [11]. Pazopanib is a TKI that targets VEGFR 1–3 and c-KIT. Its antitumor activity in advanced and progressive DTC was demonstrated in a phase II trial, which showed a partial response (PR) rate in 49% of patients (n=39). Median progression free survival(PFS) was 12 months [13,14]. XL184 (cabozantinib) is an oral inhibitor of RET, c-MET and VEGFR 1 and 2. c-MET activation triggers tumor growth and angiogenesis. Moreover, in patients with PTC and MTC, overexpression and frequent mutations of the c-MET receptor have been reported [15]. Antitumor activity of cabozantinib in patients with DTC was recently reported at the

2012 ASCO Annual Meeting. Cabanillas et al. found confirmed PR and stable disease (SD) in 53 and 40% of patients respectively (*n*=15). Disease control rate (PR + SD) was 80% at 16 weeks. At the time of data presentation, median PFS and overall survival (OS) had not been reached [16].

E7080 (lenvatinib) is also an inhibitor of multiple TK, especially VEGFRs, c-KIT, platelet-derived growth factor receptor beta stem cell factor receptor. In animal studies, it has been shown to have potent antitumor activity against small cell lung cancer and breast cancer most likely through inhibition of VEGFR-2 and VEGFR-3 [17,18]. A phase II trial of lenvatinib in RAI refractory DTC (*n*=58) by Sherman et al. showed a PR rate of 50% and a median PFS of 13 months [19]. A recent study by Rajoria et al. showed estrogen to be a key regulating factor of VEGF expression in thyroid cells, suggesting a role for anti-estrogens in the therapeutic regimen to treat thyroid cancer [20].

As a result of this increased understanding of the biological basis for thyroid cancer development, multiple clinical trials with multi-target tyrosine kinase inhibitors (TKIs) have been conducted. Sorafenib (BAY 43-9006) is an orally active TKI that targets BRAF, VEGFR 1 and 2 and RET, implementing antiangiogenic and proapoptotic actions. Results of an update of a phase II study on sorafenib, reported at the 2011 ASCO Annual Meeting by Brose et al., showed a PFS of 24 months and an OS of 35 months in 47 patients with DTC and poorly differentiated thyroid cancer (PD)(*n*=55). A PR was achieved in 38% of patients and 47% had SD [21]. An other phase II study in mainly patients with advanced DTC (*n*=30), reported a PR rate of 23%, a SD rate of 53% and a median PFS of 20 months [22]. Kloos et al. examined the effect of sorafenib mainly in patients with metastatic PTC (*n*=41) and reported a PR and SD of 15% and 56% respectively. The median PFS was 15 months [23]. Recent results of a study with the MEK 1/2 inhibitor selumetinib showed reinduction of radio iodine uptake in 60% of patients with RAI refractory thyroid cancers of follicular origin [24].

Our phase II study was designed to assess the effects of sorafenib on restoring the susceptibility to RAI therapy, tumor progression and safety in RAI refractory DTC. The first report of this study by Hoftijzer et al. showed a promising efficacy and tolerability of sorafenib, but no beneficial effects on radioiodine uptake [25]. Here, we report the long term outcomes of this sorafenib trial in advanced RAI refractory DTC with results on efficacy, safety and tolerability.

PATIENTS AND METHODS

Patients

Details of study design and patient eligibility of this study have been described previously [25]. Briefly, eligibility criteria were the presence of progressive metastases or unresectable local recurrence of DTC for which RAI therapy was no longer effective,

as indicated by prior negative post-therapeutic whole body scintigraphy (WBS). Progressive disease was defined according to Response Evaluation Criteria in Solid Tumors (RECIST) criteria 1.0 12 months before initiation of treatment. Patients had undergone total thyroidectomy and RAI ablative therapy. Eastern Cooperative Oncology Group performance status had to be less than 2 and life expectancy had to be more than 3 months. Written informed consent was provided by all patients before enrollment onto the trial. The study protocol was approved by the Institutional Review Board of the Leiden University Medical Center. This study was registered at ClinicalTrials.gov (# NCT00887107).

Study design

We performed a non-randomized, open-arm, phase II trial of sorafenib in 31 patients with advanced RAI refractory DTC. For complete description of study design we refer to the paper of Hoftijzer et al.[25]. Primary objective of this study was determination of the efficacy of sorafenib treatment with a partial response as the primary endpoint. The secondary objective was to study RAI re-uptake and the safety of sorafenib as an anti-tumor drug for advanced RAI refractory DTC patients. Sorafenib was administered at a dose of 400 mg orally twice a day until disease progression, uncontrollable side effects, death or patients own request. Safety assessments consisted of physical examination, documentation of adverse events and laboratory parameters (total blood count, electrolytes, kidney and liver function) and were performed every 4 weeks. The incidence, grade and relationship of adverse events to the drug were graded with the use of Common Terminology Criteria for Adverse Events (version 3.0).

After completion of 6 months treatment, patients were allowed to continue sorafenib treatment when a favorable response (complete remission, partial remission, or stable disease) had been achieved. Sorafenib was thereafter continued until progression according to RECIST 1.0 criteria with CT scanning performed every 6 months.

In this article, we report the long term outcomes and tolerability of sorafenib in patients with advanced RAI refractory DTC.

Statistical methods

In this study, data are reported as median ± SD, median (range) or proportions. Best objective response rate refers to the proportion of patients who had the best response rating for CR or PR according to RECIST 1.0. The OS, PFS (defined as the time from starting study drug to progression) and overall disease control rate with associated 95% CIs, were obtained using the Kaplan-Meier method. Adverse events were scored according to the National Cancer Institute's Common Terminology Criteria for Adverse Events (CTCAE), version 3.0. Other safety parameters, including

laboratory data, were summarized descriptively. Variables possibly influencing response to sorafenib were analyzed with binomial logistic regression. The calculations were performed using SPSS 17.0 for Windows (SPSS, Chicago, IL, USA).

RESULTS

Patient characteristics

All 31 patients were included in this long term outcomes study on sorafenib. The follow-up of the study ended on 1 March 2011 with 5 patients excluded from the efficacy analysis because they did not reach the first radiological evaluation at 6 months. One patient requested to stop sorafenib treatment after 4 days, 1 patient developed small cell lung cancer and 3 patients discontinued treatment because of adverse events. Median follow-up period was 25 months (range 3.5- 39 months) and median sorafenib treatment period was 9.2 months (range 0.1-39 months). By the time the follow-up of the study ended (March 2011), 4 patients (15.4%) were still on sorafenib (Figure 1).

Baseline characteristics of the included patients are listed in Table 1. As can be expected, Hurthle cell metaplasia, which is associated with poor RAI responsiveness, was overrepresented in the patient group (42%). Two of 13 PTC patients had a poorly differentiated carcinoma. A total of 15 mutations were identified on primary tumor material, of which BRAF V600E was most frequently observed with 10/13 PTC patients harboring a BRAF V600E mutation.

Figure 1 Study flowchart

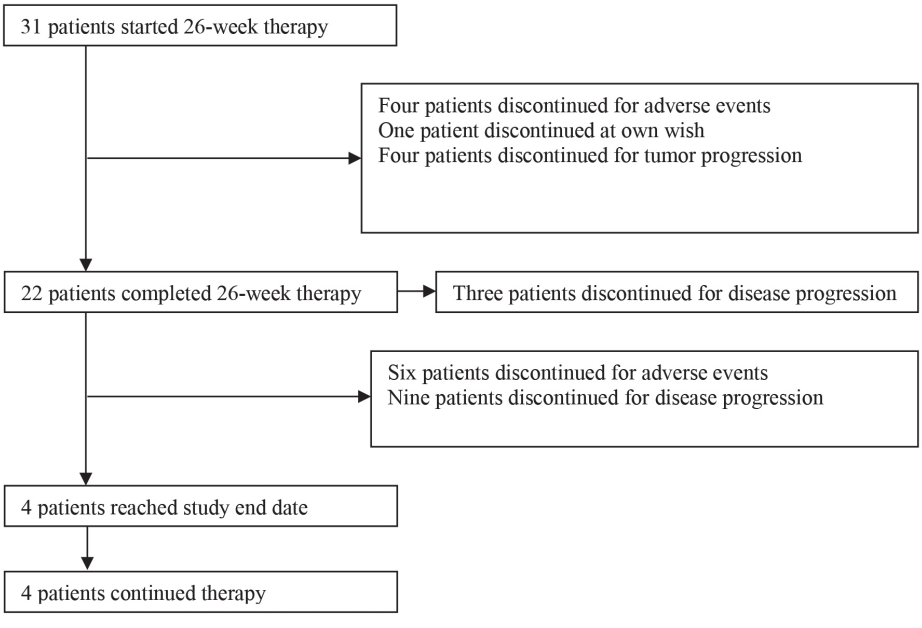


Table 2 Baseline Characteristics

	All patients (*n*=31)
Gender (No.; %)	
Female	12 (39)
Male	19 (61)
Age (year) (median, range)	64 (53-82)
Time from diagnosis (year) (median, range)	3.9 (0.3-18)
Histology (No.; %)	
Papillary	13 (42)
Tall cell	1 (3)
Hurthle cell metaplasia	2 (7)
Poorly differentiated	2 (7)
Follicular variant papillary	2 (7)
Follicular	15 (48)
Hurthle cell metaplasia	11 (35)
Mixed papillary follicular	1 (3)
Initial TNM stage (No.; %)	
IB (T2N0M0)	2 (7)
IIB (T2-3 N0-1 M0)	16 (52)
IIIA (T1-3 N1-2 M0)	5 (16)
IV (any T any N M1)	6 (19)
Unknown	2 (7)
Tumor extent at study entry (No.; %)	
Thyroid bed only	1 (3)
Lungs only	8 (26)
Lungs and bone only	8 (26)
Locally advanced* and distant metastases	9 (29)
Other	5 (16)
Mutational status (No.; %)	
BRAF	10 (32)
K-RAS	1 (3)
N-RAS	2 (6)
H-RAS	0
PIK3CA	2 (6)

* Including both thyroid bed (*n*=5) and neck lymph nodes (*n*=4)

Efficacy

Of the 31 patients enrolled in this study, 26 patients were eligible for efficacy analysis. Five patients were excluded for the reasons previously mentioned. Data on efficacy are given in Table 2 and Figure 2.

At a median follow-up 25 months, the overall disease control rate was 27%. The

total proportion of PR was 31% (n=8), which were all achieved in the first 6 months of treatment. Four patients (15%) had an ongoing partial response (PR) and 3 patients (12%) showed ongoing stable disease (SD). There were no complete responses. Individual data per patient response is summarized in Table 3. At the time of this data analysis, the cumulative number of patients with progressive disease was 15 (58%). Disease progression was not influenced by age, gender or histology, including the presence of Hurthle cell metaplasia. Furthermore, the presence of a BRAF V600E mutation was not related to disease progression. The prevalence of other mutations was too low to allow statistical analysis. Disease progression in soft-tissue metastases or bone metastases dit not significantly differ. However, the favorable responses seen in 3 patients with bone metastases were all based on regression of metastases other than bone, predominantly lung, whereas the bone metastases were stable. The overall median OS was 34.5 months (95% CI: 19-50 months). In the presence of bone metastases, median OS was significantly worse with 23 months (95% CI: 20-26 months) as compared to the median OS of the whole group. However, at the time of this data analysis, median OS was not reached for patients without bone metastases (Figure 2A). Age and gender did not influence OS. Estimation of median PFS was 18 months (95% CI: 7-29 months). Similarly, the median PFS was influenced by the presence of bone metastases. Median PFS was 20 and 12 months in the absence and presence of bone metastases respectively (Figure 2B). A relation between OS and PFS and site of bone metastases could not be established since 12 of 13 patients with bone metastases had metastases localized in the axial skeleton. Hurthle cell metaplasia and reduction of drug-dose did not influence PFS.

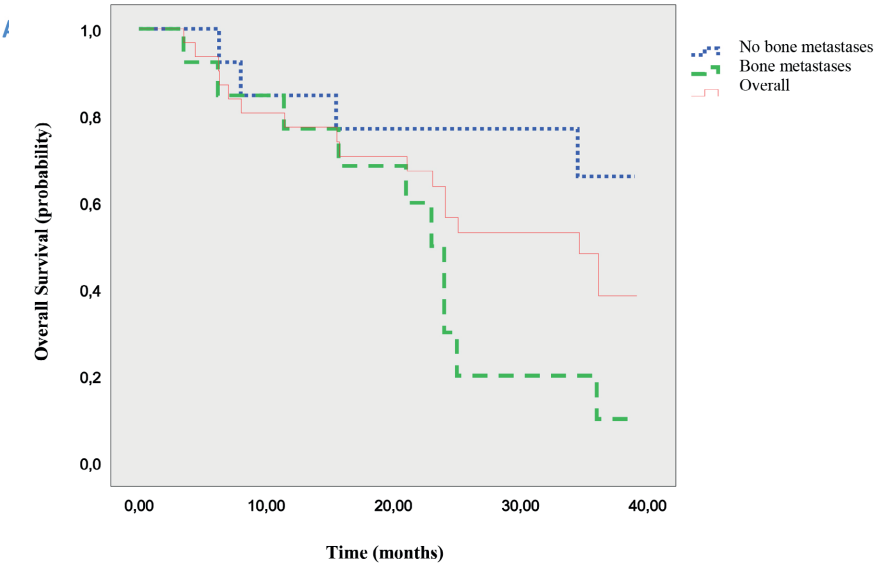
Thyroglobulin (Tg) response reflected the radiological response (Table 4). From baseline, patients with a PR had a median decrease of 24 µg/l (range -2746- 2.6 µg/l) in their serum Tg levels. Patients with SD showed a median elevation of 19 µg/l (range -60- 3724 µg/l). The serum Tg levels in patients that showed PD increased by a median of 49 µg/l (range -5320- 87736 µg/l). The 54-year-old woman with pulmonary metastases of a Hurthle cell FTC reported at 6 months with high Tg levels (99900 µg/l) but a decrease in number, size and density of all metastases, died 2 months later from disease progression with a remained high Tg level (89400µg/l).

Table 3 Efficacy analysis

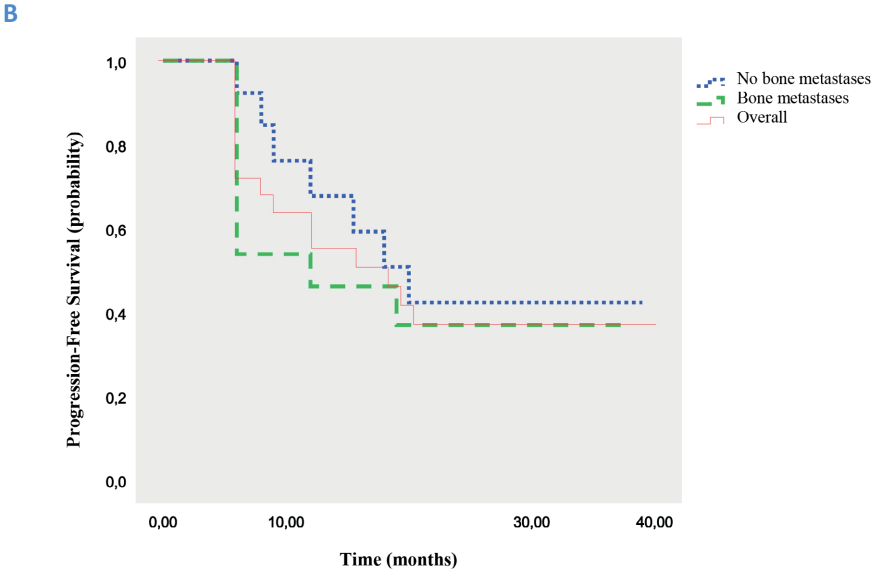
Parameter	Number of patients (% of total)
Total patients	31
Assessable patients	26
Median duration of treatment (months; range)	15 (0.1-39)
Median duration of follow up (months; range)	25 (3.5-39)
Best response by RECIST 1.0	
Complete response	0 (0)
Partial response	8 (31)†
Stable disease	3 (12)
Progressive disease	15 (58)
Overall disease control	7 (27)
Median duration of SD (months; range)	26.5 (3-38)
Median overall response duration (months; range)	29.6 (3-33)
Median PFS (95% CI)	18 (7-29)
With BM	12 (NR)
Without BM	20 (13-27)
Median OS (95% CI)	34.5 (19-50)
With BM	23 (20-26)
Without BM	NR

RECIST Response Evaluation Criteria in Solid Tumors, SD stable disease, PFS progression free survival, OS overall survival, BM bone metastases, NR not reached
† 4 of 8 patients showed an ongoing PR

Figure 2 Kaplan–Meier curves of (A) overall survival (OS) and (B) median progression-free survival (PFS)



A: Kaplan-Meier estimate of overall survival (OS). Overall median OS was 34.5 months. In the presence of bone metastases, median OS was 23 months At the time of this data analysis, median OS was not reached for patients without bone metastases.



B: Kaplan-Meier estimate of progression free survival (PFS). Estimation of median PFS was 18 months. Median PFS was 20 months in the absence and 12 months in the presence of bone metastases.

Table 3 Data on clinical benefit per patient

Response	Histology	Initial tumor stadium	Duration of response (months)
PR	Papillary	IB	33 *
PR	Follicular with Hurthle cell metaplasia	IIB	33 *
PR	Papillary	IIB	33 *
PR	Papillary	IV	30
PR	Follicular	IIB	24
PR	Follicular	IIB	24
PR	Papillary	IIB	18 *
PR	Papillary	IIIA	6
SD	Papillary	IIIA	38
SD	Follicular	IIB	36
SD	Follicular with Hurthle cell metaplasia	IIB	12

PR partial response, SD stable disease, IB (T2N0M0), IIB (T2-3 N0-1 M0), IV (any T any N M1), IIIA (T1-3 N1-2 M0), * ongoing response

Table 4 Thyroglobulin response per patient response

RECIST 1.0	CR	PR	SD	PD
Baseline serum Tg levels (median; range)	Non	50 (4.5-3126)	41 (2-1817)	382 (26.5-8570)
Last serum Tg levels (median; range)	Non	19 (0.9-1894)	94 (14-4140)	373 (38-89400)
Delta Tg (versus baseline, median; range)	Non	-24 (-2746-2.6)	19 (-60.8-3724)	49 (-5320-87736)

RECIST Response Evaluation Criteria in Solid Tumors Tg thyroglobulin, CR complete response, PR partial response, SD stable disease, PD progressive disease

Treatment tolerability and adverse events

Eighteen patients (58%) required dose reduction due to toxicity. Four of them (13%) discontinued the study later on due to adverse events (AEs).Of these, 3 patients suffered from a myocardial infarction after respectively 1, 20 and 22 months of sorafenib treatment and one patient quitted due to complaints of malaise after 39 months. Fourty-four percent of dose reductions occurred in the first month of treatment. Further details on dose reduction and daily sorafenib dose are listed in Table 5. In total, 7 patients (23%) terminated study participation as a result of drug-

related AEs. There were 3 patients who discontinued sorafenib before dose reduction after 1, 12 and 19 months due to angioedema, congestive heart disease, and malaise and weight loss, respectively. One patient discontinued treatment after 3 months due to the development of small cell lung cancer. One patient was diagnosed with carcinoma of the tongue after 46 months of sorafenib treatment. Thirteen patients (42%) required levothyroxin dose adjustments in order to maintain euthyroidism.

All observed AEs are listed in Table 6. Treatment-related AEs were predominantly of grade 1 or 2, with the most common events including hand foot syndrome (HFS), weight loss, diarrhoea and rash. Grade 3 AEs consisted of HFS (23%), weight loss (10%), diarrhoea (6%), rash (16%), mucositis (10%) and congestive heart disease (3%). Grade 4 AEs comprised myocardial infarction in 3 patients (10%), which led to death within 2 months after the infarction in one patient. The majority of AEs were seen in the first year of treatment and were controllable with either dose reduction, medication or supporting measures (f.e. dietary consultation and additional feeding). No correlations were found between toxicity and performance state or age.

Table 5 Daily sorafenib dose and dose reduction

	3 months	**6 months**	**12 months**
Dose reduction (No.; %)	13 (42)	16 (52)	18 (58)
Daily sorafenib dose (mg)			
Mean (± SD)	671 (±198)	584 (±230)	562 (±175)
Median (range)	600 (200-800)	600 (200-800)	600 (400-800)

Table 6 Adverse events

	All	Grades Number of patients (% of category)			
Event	**Number of patients (% of total (*n*=31))**	**1**	**2**	**3**	**4**
Hand foot syndrome	22 (71)	8 (36)	7 (32)	7 (32)	
Weight loss	18 (58)	7 (39)	10 (56)	3 (17)	
Diarrhoea	16 (52)	5 (31)	9 (56)	2 (13)	
Rash	17 (55)	11 (65)	1 (6)	5 (29)	
Alopecia	16 (52)	14 (88)	2 (12)		
Mucositis	15 (48)	11 (73)	1 (7)	3 (20)	
Hypocalciemia	15 (48)	14 (93)	1 (7)		
Hypertension	13 (42)	4 (31)	4 (31)	5 (38)	
Hypophosphatemia	11 (35)	11 (100)			
Anemia	11 (35)	11 (100)			
Hypoparathyroidism	10 (32)		10 (100)		
Thrombopenia	9 (29)	9 (100)			
Hypothyroidism	8 (26)		8 (100)		
Leukopenia	7 (23)	7 (100)			
Nausea	3 (10)	3 (100)			
Myocardial infarction	3 (10)				3 (100)
Congestive heart disease	1 (3)			1 (100)	
Hematuria	1 (3)		1 (100)		
Deep venous thrombose	1 (3)			1 (100)	
Hyponatriemia	1 (3)	1 (100)			
Pneumothorax	1 (3)		1 (100)		
Small cell lung cancer	1 (3)				1 (100)

According to Common Terminology Criteria for Adverse Events (version 3).

DISCUSSION

The developments in the treatment of advanced thyroid cancer are a result of increased understanding of thyroid tumorigenesis. A high percentage of differentiated thyroid cancers contain aberrations in the RET/PTC-RAS-RAF-MAPK pathway. In addition, the RET–RAS–RAF pathway leads to VEGF and VEGFR synthesis through the EFGR activated cascade. Hence, compounds targeting the activated RET–RAS–RAF pathway and beyond, anti angiogenic compounds or a combination of both, may be effective in RAI non-avid DTC [26]. Several clinical trials in thyroid cancer have shown considerable percentages of clinical responses, suggesting this assumption to be accurate. Of the targeted therapies that have shown promising results in advanced differentiated thyroid cancer, only sorafenib targets both BRAF and RET, as well as VEGFR. However, sorafenib is a relatively weak BRAF inhibitor. The most potent BRAF inhibitor, vemurafenib, might be more effective in PTC harboring a BRAF mutation and is currently under study in an international phase II trials (www.clinicaltrials.gov, NCT01286753) [27].

Our study is the first study reporting the long term effects of sorafenib treatment in advanced RAI refractory DTC patients. We also provided results of the influence of bone metastases on survival outcome and PFS. Our results were not influenced by subtypes of thyroid cancer, since we only included patients with DTC, ensuring a homogeneous study population. In our trial we observed beneficial effects of sorafenib. At 6 and 25 months, median PFS was 14.5 and 18 months respectively. At 6 and 25 months, median OS was 25 months and 34.5 months respectively. The PR rate of 25% we observed in the first 6 months of the trial is one of the highest PR rates reported in sorafenib trials in DTC. Kloos et al. found a PR rate of 15% in patients with metastatic PTC [23]. Gupta et al. reported a PR of 23% (DTC, MTC and ATC) whereas Ahmed et al. observed a PR in 21% of patients with metastatic thyroid cancer (DTC and MTC) [22,28]. Our high PR rate could be attributed to the differences in patient categories (histologies, tumor stages, sites of metastases and tumor extent), study design and analytical methods between the different phase II trials.

The explanation for the significant less favorable outcome in patients with bone metastases remains unclear. The relationship between the genetic profile of bone metastases and responsiveness to sorafenib is hard to establish given the lower tissue levels and difficulties in obtaining tissue from bone metastases. It can be hypothesized that VEGFR targeted therapies are less effective in bone metastases since the role of VEGFR signal transduction in tumor expansion may differ between soft tissue and bone metastases. Another explanation may be that through systemic release of cytokines or proteins from the bone microenvironment the presence of bone metastases influences the response to sorafenib in soft-tissue metastases. The dose of sorafenib used in our study was generally well tolerated, although dose reductions were required

in 58% of patients. Tumor response however, was not affected by dose reductions. Toxicities observed in our study were mostly grade 1-2 and similar to those reported in the other phase II trials of sorafenib [21-23,28,29]. A long term safety evaluation of sorafenib in advanced renal cell carcinoma showed similar results, supporting our findings [30]. Dermatologic adverse events were the most commonly observed. A recent study of sorafenib in patients with hepatocellular carcinoma reported better response in patients with early skin reactions [31], however, we were not able to establish that correlation. Adverse effects were in the majority of cases manageable with dose reduction, medication or supplementary measures. However, beneficial effects on tumor response should always be weighed against the side-effects and quality of life of targeted therapies [3,4,29].

There are some issues that have to be addressed to. RECIST criteria should be applied for judgement of progression for inclusion in trials and for follow-up scans to determine treatment effect. However, RECIST criteria have several limitations for the determination of efficacy of targeted drug activity. This is shown by the fact that in our study, some of the patients with PD based on new lesions had stable or even reduction in the target lesion's longest diameter. Nevertheless, the RECIST criteria are the best criteria to date and should be used in every clinical trial.

Another issue is that in studies of targeted therapy in advanced thyroid cancer, a substantial proportion of patients (33%-50%) had stable disease of varying duration as best response [32]. Given the indolent natural history of these tumors, stable disease may be considered to be of limited value. Considering this, the determination of the best primary end point stays an issue. Objective response rates do not correlate per se with OS. Given the indolent nature of advanced thyroid cancer, progression-free survival or OS are not always preffered primary end points. Therefore, only patients with documented progressive disease should be included in clinical trials, permitting a smaller sample size and a shorter time for follow-up to demonstrate a difference in response to therapy between treatment groups. In addition, sorafenib treatment should only be initiated in patients with progressive disease or significant tumor burden in the presence of RECIST targets. Furthermore, patients with stable disease at baseline may not benefit from therapy and do not need treatment with targeted agents, with all possible side-effects.

In conclusion, sorafenib is generally well tolerated and has demonstrated to be clinically active in the long term in advanced RAI refractory DTC. In order to enhance sorafenib treatment efficacy, further trials need to focus on sequential or combination therapy, since patients eventually become progressive on sorafenib or do not tolerate it. More trials with new targeted agents are under way and the outcomes of these trials will contribute to a better treatment and understanding of advanced thyroid cancer.

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3

(SECONDARY) SOLID TUMORS IN THYROID CANCER PATIENTS TREATED WITH THE MULTI-KINASE INHIBITOR SORAFENIB MAY PRESENT DIAGNOSTIC CHALLENGES

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ABSTRACT

Background

Sorafenib is an orally active multikinase tyrosine kinase inhibitor (TKI) that targets B-type Raf kinase (BRAF), vascular endothelial growth factor receptors (VEGFR) 1 and 2, and rearranged during transfection (RET), inducing anti-angiogenic and pro-apoptotic actions in a wide range of solid tumors. A side effect of sorafenib is the occurrence of cutaneous squamous tumors.

Case presentation

Here we describe three patients with a history of sorafenib treatment for advanced radioactive iodine refractory papillary thyroid cancer (two with a *BRAF* c.1799 T>A and one carrying a rare c.1799-1801het_delTGA mutation) who presented with secondary non-cutaneous lesions. The first patient was diagnosed with a squamous cell carcinoma (SCC) of the tongue, the second patient with a primary adenocarcinoma of the lung, and the third with a SCC originating from the cricoid. Secondary analysis was required to show that the latter two presentations were in fact recurrent thyroid cancer.

Conclusion

These findings suggest that drugs such as sorafenib may induce metaplasia/clonal divergence of metastatic thyroid cancer and thus cause diagnostic misclassification. Furthermore, sorafenib is potentially involved in the tumorigenesis of secondary non-cutaneous SCC. These observations should now be confirmed in larger series of patients treated with drugs such as sorafenib.

Keywords

Sorafenib, differentiated thyroid cancer, squamous cell carcinoma, squamous differentiation, clonal divergence

BACKGROUND

Sorafenib (BAY-43-9006) is an orally active multikinase tyrosine kinase inhibitor (TKI) that activates anti-angiogenic and pro-apoptotic pathways, targeting the *B-type Raf kinase* (BRAF), vascular endothelial growth factor receptors (VEGFR) 1 and 2, and *rearranged during transfection* (RET). Sorafenib is widely approved for the treatment of patients with hepatocellular carcinoma (HCC) and advanced renal cell carcinoma (RCC) in well-defined phases of disease. From June this year sorafenib will also be registered for the treatment of patients with thyroid cancer by the European Medicines Agency (EMA). Multiple clinical trials have been conducted in a wide range of cancers (lung, thyroid, breast, colorectal) using sorafenib as a single agent or in combination treatment (www.clinicaltrials.gov).

Recently published results of a phase III trial of sorafenib in patients with advanced radioactive iodine 131-I (RAI) refractory differentiated thyroid carcinoma (DTC) showed that sorafenib has clinically relevant antitumor activity and a generally well-tolerated profile of adverse events (AEs). The most commonly reported sorafenib-related AEs in DTC include hand-foot syndrome, hypertension, weight loss, diarrhea and rash.(1,2) Recent reports have also suggested a possible causal link between sorafenib therapy and the development of cutaneous squamous cell carcinomas (SCC).(3-8)

We now describe three patients who received sorafenib during treatment for advanced RAI refractory DTC and presented with secondary non-cutaneous squamous lesions.

MATERIALS AND METHODS

The presence of somatic DNA mutations including *BRAF* (V600E and V600K), *KRAS* (codon 12/13), and *PIK3CA* (exons 9 and 20) were determined by quantitative real-time PCR (qPCR) with hydrolysis probes (Custom TaqMan® Assay Design Tool, Applied Biosystems, Nieuwerkerk a/d IJssel, NL), and when indicated, by standard Sanger DNA sequencing on an ABI 3739 automated sequencer (Applied Biosystems, Foster City, CA, USA).(9) Tissues were microdissected to enrich for tumor cells and tumor areas were selected based on the analysis of a hematoxylin eosin (HE)-stained tissue slide. Tumor DNA was subsequently isolated using the Nucleospin Tissue kit (Marcherey-Nagel, Bethlehem, PA, USA) according to manufacturer's protocol.

PATIENTS

Patient 1

A 67-year-old female was diagnosed with a well differentiated papillary thyroid carcinoma (PTC, *BRAF* c.1799 T>A; p.V600E mutation positive), stage T2N0M0, in 1989. She underwent a total thyroidectomy and RAI ablation therapy. Local recurrent disease was diagnosed ten years later and treated with a left-sided modified radical neck dissection, followed by RAI therapy. The post-therapy scintigraphy showed no RAI uptake. Six years later routine follow-up with computed tomography (CT) identified metastatic disease with multiple lung lesions, the largest measuring 5 mm (2005). A total body scintigraphy after RAI therapy showed no uptake of RAI despite elevated thyroglobulin levels (68 ug/l), indicating RAI refractory disease. Due to the progression of disease two years later (2007) the patient received sorafenib (2 x 400 mg/day initially, reduced to 1 x 200 mg/day after 6 months) in the context of a phase II study.(2,10) Follow-up showed stable disease according to the Response Evaluation Criteria in Solid Tumours (RECIST) 1.0.(11) Thirty-nine months after starting sorafenib, with ongoing stable disease, the patient stopped therapy mainly due to diarrhea.

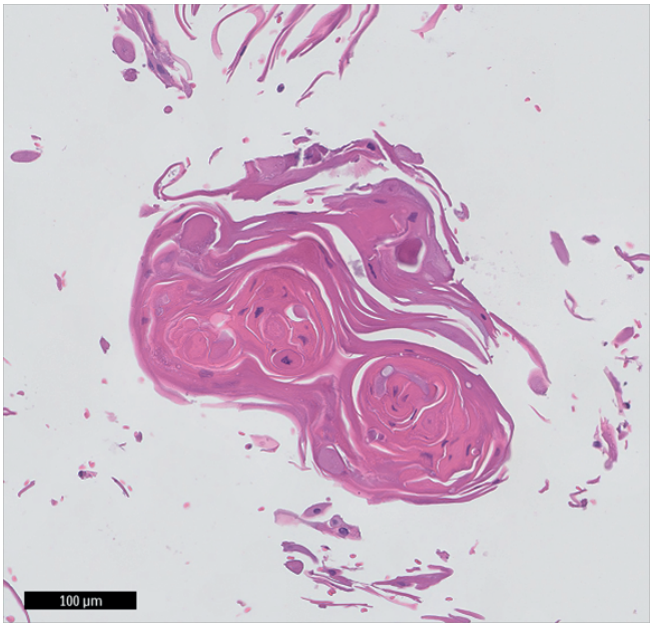
After starting sorafenib treatment, the patient developed several skin lesions (for a summary see Table 1). Furthermore, within 2 months of beginning sorafenib therapy the patient developed left-sided tongue complaints, originally histologically diagnosed and treated as mucosal hyperplasia with Candida albicans infection. The symptoms persisted however and eventually led, 46 months after the initial complaints, to the diagnosis of a T2N2cM0 functional irresectable SCC of the tongue (*thyroid transcription factor* (TTF)-1 and thyroglobulin immunohistochemically negative; *BRAF* p.V600E negative) with ipsi-lateral lymph node metastasis (Figure 1). Despite chemo-radiation therapy with 7 rounds of cisplatin, the patient died 5 months after diagnosis of the SSC.

Table 4

Date	Lesion
February 2008	SCC on the back Candida infection of the tongue
April 2008	Reactive epithelial skin lesion on the back without obvious atypia Leukokeratosis of the tongue with atypia and inflammation
June 2008	Lesion with inverted follicular keratosis on the lower left leg
September 2009	Trichilemmoma of the nose
March 2010	Irritated verruca seborrhoica upper right leg
May 2010	Reactive epithelial hyperplasia of the tongue due to a candida infection
August 2011	Invasive squamous cell carcinoma of the tongue Multiple lymph node metastasis of the SCC in the neck region
December 2011	SCC lymph node metastasis in the left axilla

Summary of lesions seen in patient 1 after starting sorafenib treatment November 2007.

Figure 1 Lymph node metastasis of the SCC of the tongue in patient 1

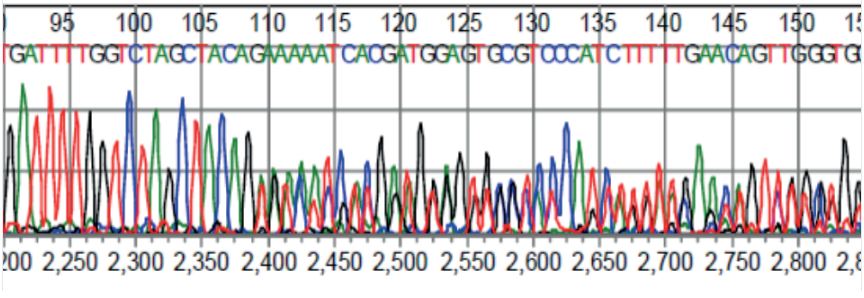


HE staining of a fine needle aspirate (FNA) of a cervical lymph node using a standard embedding procedure of cytology material and histologic processing. Atypical squamous cells with (para-) keratotic horn were seen, indicative of metastasized SCC.

Patient 2

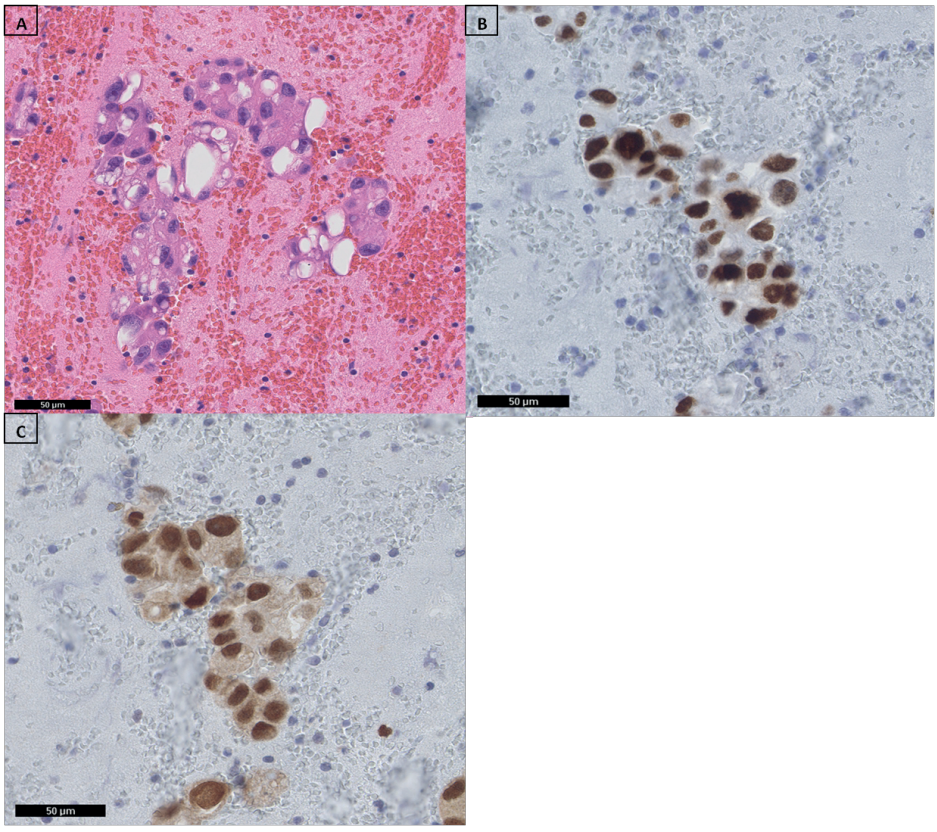
The second (male) patient was diagnosed with a T4N1Mx PTC without squamous metaplasia (positive for the rare *BRAF* mutation c.1799-1801het_delTGA, Figure 2) at the age 67.(12-14) He underwent a total thyroidectomy with a right-sided lymph node dissection, followed by RAI ablation therapy in 2001. In 2004 the patient had recurrent disease, and a bilateral para-tracheal lymph node dissection was performed. A year later he presented with local recurrent disease and the presence of multiple pulmonary metastases. A whole body scintigraphy after RAI therapy showed no RAI uptake, while thyroglobulin levels were 133 ug/l, thus demonstrating RAI refractory disease. In 2007, the patient was referred to our hospital for inclusion in a phase II trial and received sorafenib 400mg twice daily.(2,10) Despite initial stable disease, the patient became progressive under sorafenib after 19 months of therapy. The patient was subsequently enrolled in a clinical study (RAD001, www.clinicaltrials.gov CRAD001CNL08T) to determine the efficacy of everolimus in patients with progressive irresectable recurrent or metastatic differentiated, undifferentiated (anaplastic) and medullary thyroid carcinoma. The patient ceased everolimus therapy 18 months later due to progressive disease. A CT showed a new pulmonary lesion and a number of (non)target lesions according to RECIST 1.0.(11) Since all conventional or study-based treatment options had been exhausted, the patient was referred back to his own hospital where he presented 2 months later with progressive dyspnea due to malignant pleural effusion. A CT showed multiple bilateral pulmonary metastases and a large right para-tracheal lesion. Surprisingly, right-sided pleural fluid cytology elsewhere revealed a primary adenocarcinoma of the lung. Immunohistochemistry of embedded cytological material showed TTF-1 and *cytokeratin* (CK) 7 positivity and absence of staining for thyroglobulin, *cluster of differentiation* (CD) 56 and CK20. Additional *paired box* (PAX) 8 and CK19 staining in our institute were however positive, indicative of metastatic PTC (Figure 3). Molecular testing confirmed this diagnosis with the presence of the previous identified *BRAF* mutation. The patient died a month later due to disease progression.

Figure 2 *BRAF* DNA sequencing



Identification of a rare *BRAF* mutation c.1799-1801het_delTGA.

Figure 3 Pathological analysis of pleural effusion in patient 2



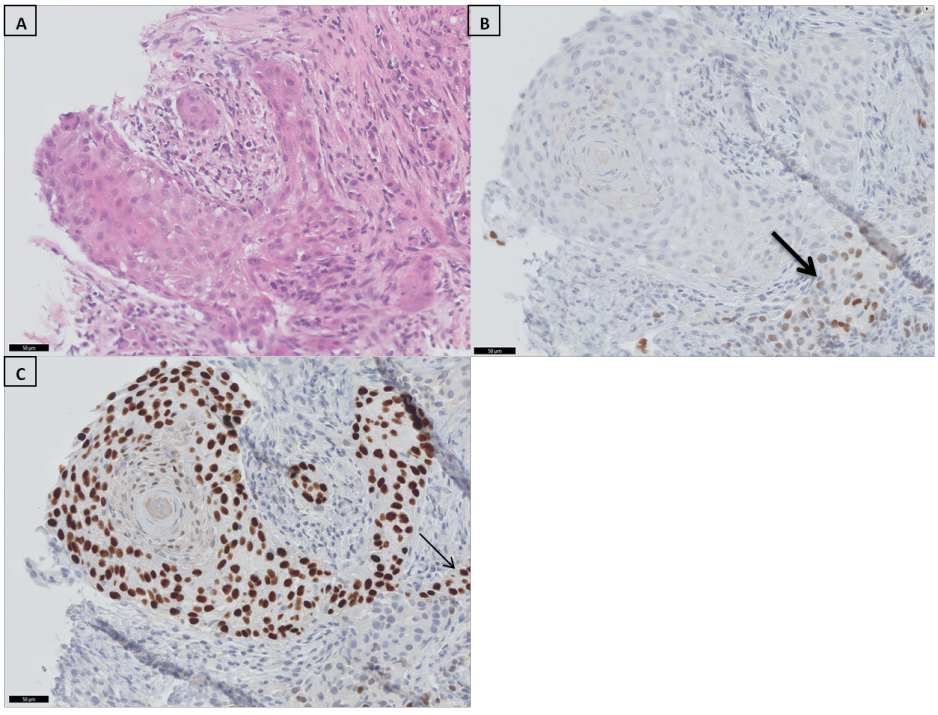
FNA of a right-sided pleural effusion after embedding and histological processing.
A: HE; B: TTF1 immunohistochemistry (IHC); C: PAX8 IHC

Patient 3

A 60-year-old male was diagnosed with a T2N0M0 poorly differentiated PTC (*BRAF* c.1799 T>A; V600E mutation positive) in 2002. The patient was treated by total thyroidectomy with a left-sided lymph node dissection, followed by RAI ablation therapy. Three years later (2005) he presented with multiple pulmonary lesions, non-RAI avid on whole body scintigraphy after RAI therapy, and thyroglobulin levels of 127 ug/l. Due to disease progression in 2008 the patient was included in a phase II trial (and received 2 x 400 mg sorafenib daily).(2,10) In the first 6 months a partial response was achieved, which was ongoing until therapy discontinuation 32 months later due to complaints of diarrhea and hand-foot syndrome. A CT four months after therapy discontinuation still showed an ongoing partial response per RECIST 1.0.(11) However, seven weeks later the patient presented with respiratory failure due to airway obstruction. A large tumor originating from the cricoid was visible on CT scan. Initial histological examination

elsewhere concluded that the lesion was a SCC originating from the cricoid. Revision of the tumor showed a lesion with squamous differentiation (Figure 4). Although P63 staining (indicative of squamous differentiation) was positive in a subset of cells, immunohistochemistry also showed TTF-1 and thyroglobulin to be focally positive, indicative of a PTC recurrence. Surprisingly, the *BRAF* mutation present in the primary tumor was not recovered from this tumor, possibly suggesting clonal divergence after sorafenib treatment. Given the fact that local treatment was not an option the patient received a tracheal stoma followed by radiotherapy. He died 8 months later due to tumor progression.

Figure 4 PTC recurrence in the laryngeal cricoid of patient 3



In a biopsy from the laryngeal cricoid solid sheets of tumor cells were seen with focal keratinisation. Panel A shows the HE stained tissue. Immunohistochemical analysis of TTF-1 (panel B) and P63 (Panel C) is shown. A large proportion of the cells stained positive for P63, supporting the squamous features. However, there was a fraction a cells that stained moderately positive for TTF-1 (thick arrow in panel B), partly overlapping with P63 positivity (thin arrow in panel C). Thyroglobulin also stained focally positive (not shown). Due to the TTF-1 and thyroglobulin positivity, we favoured the diagnosis of recurrent papillary thyroid cancer with remarkable squamous metaplasia over a primary laryngeal squamous carcinoma.

DISCUSSION

The cases described here present three interesting observations. Firstly, (although lacking proven causality) our demonstration of a primary tongue carcinoma that arose after long-term sorafenib treatment suggests that treatment with the multikinase inhibitor sorafenib may cause other effects additional to squamous skin lesions.(3-8) However, since patient 2 was subsequently treated with everolimus, a combined effect of sorafenib and everolimus cannot be ruled out. Secondly, we note that metaplastic changes or clonal divergence can result in the misdiagnosis of recurrences of thyroid cancer. Indeed, clonal divergence at the molecular level might be an aspect of PTC with *BRAF* c.1799 T>A; p.V600E mutations. Thirdly, we identified a rare *BRAF* mutation, c.1799-1801het_delTGA, which will be missed by the allele-specific assays currently in use in daily practice in many laboratories.

It has been proposed that *BRAF* kinase inhibitors can stimulate proliferation in cells lacking mutant *BRAF* via a paradoxical activation of RAF-MEK-ERK1/2 pathway signaling in the presence of upstream activation of RAS, thus possibly explaining the development of cutaneous SCC.(3-8) This hypothesis was supported by a study that analyzed oncogenic mutations in cutaneous SCCs in melanoma patients treated with the *BRAF* inhibitor vemurafenib. This study found that 13 out of 21 tumors harbored a RAS mutation.(15) Furthermore, there are several reports on patients treated with a *BRAF* inhibitor who developed secondary tumors. One case has been described of a patient on vemurafenib treatment for a melanoma who developed a RAS-mutant leukemia correlating with enhanced extracellular signal-regulated kinases (ERK) signaling.(16). Another study reports the development of four colonic adenomas, one hyperplastic colonic polyp and six gastric polyps in a patient also treated with vemurafenib for a melanoma. However, no evidence of RAS mutations was reported in any of the adenomas identified in this patient.(17). In addition, progression of a previously present KRAS mutated colon carcinoma due to stimulated ERK signaling was reported in a patient with a melanoma treated with dabrafenib.(18) A similar molecular explanation might be relevant to the cases we now present, although the presence of RAS mutations was not completely tested in the lesions with squamous metaplasia.

In summary, we described three patients with advanced RAI refractory DTC and a history of sorafenib treatment who presented with a secondary non-cutaneous lesion. The first patient was diagnosed with a SCC of the tongue; the remaining two patients had recurrent disease with altered morphology and molecular biology suggesting clonal divergence, with initial diagnoses as a primary adenocarcinoma and SCC, originating from the lung and cricoid respectively. Although the possibility that these tumors were already present in the form of micrometastases prior to sorafenib treatment cannot be excluded, these observations are quite remarkable. It is therefore important that a pathologist is familiar with the patient's medical history and treatment and that the possibility of unusual presentation is kept in mind when considering secondary lesions.

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PART 2
EVEROLIMUS

4

BENEFICIAL EFFECTS OF THE MTOR INHIBITOR EVEROLIMUS IN PATIENTS WITH ADVANCED MEDULLARY THYROID CARCINOMA; SUB-GROUP RESULTS OF A PHASE II TRIAL

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Int J Endocrinol. 2015; 348124

The medication used in this trial was provided by Novartis. Furthermore, Novartis financially supported the conduction of this trial

ABSTRACT

Objective

Until recently, advanced medullary thyroid cancer (MTC) had few treatment options except surgery. Everolimus is a rapamycin derivative that targets mTOR, and has shown encouraging results in neuroendocrine tumors both in vitro and in vivo. As part of a prospective phase II clinical trial, we analyzed the safety of everolimus and efficacy on tumor progression in advanced MTC.

Methods

Seven patients with per RECIST 1.1 documented progressive metastatic MTC, were included and received everolimus 10mg daily. The primary objective was to determine treatment efficacy. Secondary endpoints included progression-free survival (PFS), overall survival (OS), toxicity and pharmacokinetics (PK).

Results

The median duration of follow-up was 28 weeks (17-147). Five patients (71%) showed SD, of which 4 (57%) for >24 weeks. Median PFS was 33 weeks (95%CI:8-56), and median OS was 30 weeks (95%CI:15-45). Toxicity was predominantly grade 1 or 2 and included mucositis (43%), fatigue (43%) and hypertriglyceridemia (43%). Four MTCs (57%) harbored the somatic *RET* mutation c.2753T>C,p.Met918Thr. The best clinical response was seen in a MEN2A patient. The PK characteristics were consistent with data from phase I studies. One patient exhibited extensive toxicity accompanying elevated everolimus plasma concentrations.

Conclusions

This study suggests that everolimus exerts clinically relevant antitumor activity in patients with advanced MTC. Given the high level of clinical benefit and the relatively low toxicity profile, further investigation of everolimus in these patients is warranted.

Keywords

Medullary thyroid cancer, everolimus, phase II, clinical trial, targeted therapy

INTRODUCTION

Medullary thyroid cancer (MTC) is a neuroendocrine tumor derived from the calcitonin-producing thyroid C cells and accounts for 3-5% of cases of thyroid cancer [1]. Until recently, besides surgery few curative and palliative treatment options were available for patients with MTC. Conventional treatment modalities have disappointing outcomes in most patients with MTC, indicating the need for new treatments.

The American Thyroid Association therefore recommends that these patients should be enrolled in clinical trials. Concurrently, an increased understanding of thyroid tumorigenesis has led to the identification of potential targets, with novel therapeutic agents now able to target these biological abnormalities.

In addition, many genetic alterations affecting tyrosine kinase signaling pathways have recently been identified in various forms of thyroid cancer such as the activating *RET* mutations present in > 95% of hereditary MTC and in 20-50% of sporadic MTC [2,3]. The phosphatidylinositol-3-kinase (PI3K)/Akt pathway regulates cell growth, proliferation and survival in all thyroid tumor subtypes [4]. An important effector in the PI3K/Akt pathway is the mammalian target of rapamycin (mTOR). Activation of the mTOR serine/threonine protein kinase has been reported in a variety of malignant tumors, including thyroid tumors, with an estimated 70% of all tumors showing mTOR up-regulation [5].

As a result of this accrued understanding of the biological basis of thyroid cancer development, several clinical trials with multi-target tyrosine kinase inhibitors (TKIs) have been conducted. Everolimus is an orally available derivative of rapamycin, targeting mTOR. Everolimus exerts its activity through high affinity interaction with an intracellular receptor protein, the immunophilin FKBP12. The FKBP12/everolimus complex subsequently interacts with the mTOR protein kinase, inhibiting downstream signaling events involved in regulation of the G1 to S-phase transition[6]. The use of everolimus in neuroendocrine tumors has shown encouraging results, both in vitro and in vivo [7-9].

We initiated a phase II study to assess safety and efficacy of everolimus on tumor progression in patients with advanced thyroid carcinoma (THYRRAD, www.clinicaltrials.gov CRAD001CNL08T). In addition, pharmacokinetic parameters were assessed. Here we present a subgroup analysis of seven MTC patients.

MATERIALS AND METHODS

Patients

Eligibility criteria were the presence of per RECIST 1.1 (Response Evaluation Criteria In Solid Tumors) documented progressive metastatic or inoperable MTC in the 12 months prior to therapy [10]. Patients were required to be ≥ 18 years of age with a Karnofsky performance score > 70%. Laboratory requirements consisted of adequate bone marrow function (absolute neutrophil count ≥ 1,500/μL, platelets ≥100,000/μL, hemoglobin ≥ 5.6mm/L), liver function (serum bilirubin ≤ 1.5× upper limit of normal (ULN), serum ALT and AST ≤ 2.5× ULN) and renal function (serum creatinine ≤ 2× ULN). Women of childbearing age were required to have a negative serum or urinary pregnancy test within 14 days prior to the first dose of the study drug. Patients were not excluded based on number or type of prior therapies received, with the exception of prior targeted therapy with everolimus or other mTOR inhibitors.

Written informed consent was provided by all patients before enrollment in the trial. The study protocol was approved by the Institutional Review Board of Leiden University Medical Center and performed in the university hospitals of Leiden and Groningen. This study was registered at ClinicalTrials.gov (#NCT01118065).

Study design

We performed a non-randomized, open-label, multi-center, single arm phase II trial of everolimus in patients with advanced thyroid cancer; 28 patients with differentiated (DTC), 7 patients with anaplastic (ATC) and 7 patients with medullary (MTC) thyroid carcinoma were included. The primary objective was to determine the efficacy (response rate and stable disease >24 weeks) of everolimus. Secondary objectives were determination of the maximum percentage of tumor reduction for target lesions, describing activity time to event endpoints and assessment of toxicity, adverse events (AEs) and pharmacokinetics. Eligibility assessments, including a review of medical history and prior treatments, physical examination, and disease staging assessments were performed within 4 weeks prior to the first dose of everolimus. Baseline evaluations, comprising performance status, vital signs and laboratory tests were assessed within 2 weeks prior to initiation of therapy. Everolimus was administered at a dose of 10 mg orally once daily until disease progression, unacceptable toxicity, death, or patients' own request. Objective tumor response and time of progressions was measured according to the RECIST criteria version 1.1 every 12 weeks (± 2 weeks) during the first year, thereafter every 6 months and at study discontinuation.

Safety assessments were made every 4 weeks by adverse event collection, standard clinical and laboratory tests, and physical examinations. In case of toxicity or AEs requiring dose adjustments, drug dosing was interrupted or modified according

to the guidelines. AE monitoring was continued for at least 4 weeks following the last dose of study treatment. The incidence, grade and casual relationship of adverse events were graded with the use of Common Terminology Criteria for Adverse Events (CTCAE, version 4.0). Following study discontinuation, all patients were followed for survival for 4 weeks.

Laboratory parameters

Serum thyroid stimulating hormone (TSH), free thyroxine (T4), carcinoembryonic antigen level (CEA), calcitonin and safety parameters were assessed at all visits. Safety parameters included a total blood count as well as serum levels of sodium, potassium and creatinine, lipids, coagulation and renal and liver function.

Somatic mutation spectrum screening

DNA for mutation analysis was available for six MTC tumors. Somatic hotspot mutations were identified using a custom Ampliseq panel that targets somatic hotspot mutations in 22 genes using Ion Torrent Ampliseq sequencing chemistry (Life Technologies, Foster City, CA. Details available on request). The samples were sequenced using the Ion Torrent PGM (Life Technologies, Foster City, CA). FastQ sequence data files from the Ion Torrent PGM were analyzed with NextGENE™ software (version v.2.3.4.2, Softgenetics, State College, PA) using standard settings for somatic mutation analysis, excluding known polymorphisms.

Pharmacokinetics

In order to measure blood concentration levels of everolimus and assess everolimus steady-state pharmacokinetics (PK), patients were admitted to hospital for PK sampling on day 15 of each treatment cycle. Samples were collected in EDTA tubes at pre-dose and at 1, 2, and 3 hours after everolimus intake. Additional PK sampling at 4, 5, 6, 7 and 8 hours after everolimus intake was optional. Everolimus concentrations in whole blood were determined using a validated Ultra Performance Liquid Chromatography - Tandem Mass Spectrometric (UPLC-MS/MS) assay. Pharmacokinetic parameters were calculated with a non-compartmental approach using WinNonLin and included the Area Under the Concentration-time curve over the dosing interval ($AUC_{0\text{-}24hr}$), trough everolimus concentration (C_{trough}), time to reach peak concentration (T_{max}), peak concentration (C_{max}) and the elimination half-life ($T_{½}$).

Statistical analysis

Seven MTC patients were analyzed as a separate cohort for response rate. If no responses were present in this patient group, we could conclude that further investigation of everolimus in MTC patients is unwarranted. Endpoints were reported as median (range)

or proportions. Estimates of progression-free survival (PFS) (time from starting study drug to progression or death, whichever occurred first) and OS (time from starting study drug to the date of death by any cause), with associated 95% CIs, were obtained using the Kaplan-Meier method. Patients who were progression-free and/or alive at the time of data analysis were censored. Variables influencing the response to everolimus were analyzed with binominal logistic regression. The calculations were performed using SPSS 20.0 for Windows (SPSS, Chicago, IL, USA).

RESULTS

Patient characteristics

All 7 patients with medullary thyroid carcinoma were included in this efficacy and tolerability analysis. The follow-up of the study ended on 31 December 2013 with a median follow-up of 28 weeks (range 17-145 weeks). The median everolimus treatment period was 17 weeks (range 6-116 weeks). One patient was still on everolimus treatment at the time follow-up ended.

Baseline characteristics are listed in table 1. Two (29%) females and 5 (71%) males were included, with a median age of 53 years (range 44-74). At study entry, 1 (14%) patient had locally advanced disease, while the other 6 (86%) had distant metastasis. None of the patients were treatment-naïve.

Efficacy

Efficacy analysis showed promising results. Five patients (71.4%) showed stable disease (SD), with 4 (57.1%) having SD lasting >24 weeks. Median SD duration was 24 weeks (range 17-117 weeks). At the time of data analysis (31-12-2013), 1 patient still had ongoing SD. There were no complete (CR) or partial (PR) responses. Data on efficacy are given in table 2 and figure 1.

Estimated median PFS was 33 weeks (95% CI: 8-56 weeks). The median overall survival (OS) was 30 weeks (95% CI: 15-45 weeks). Disease progression and survival appeared not to be influenced by age, gender, disease site, mutational status, everolimus blood concentration or dose reduction. Changes in calcitonin and CEA could not be related to clinical outcome, as demonstrated in table 3 and figure 2.

Table 1 Baseline characteristics

	All patients (*n*=7)
Gender (*n*, %)	
Female	2 (29)
Male	5 (71)
Age (year; median, range)	53 (44-74)
Time from diagnosis (year; median, range)	4.3 (1.6-25.6)
Initial TNM stage (*n*, %)	
IB (T2N0M0)	0
IIB (T2-3 N0-1 M0)	2 (29)
IIIA (T1-3 N1-2 M0)	3 (43)
IV (any T any N M1)	2 (29)
Unknown	0
Tumor extent at study entry (*n*, %)	
Locally advanced	1 (14)
Metastatic	6 (86)
No. of disease sites (*n*, %)	
1	1 (14)
2	2 (29)
≥3	4 (57)
Mutational status (*n*, %)	
RET M918T	4 (57)
MEN-IIA	1 (14)
EGFR P848L	1 (14)
Unknown	1 (14)
Prior treatment (*n*, %)	
Surgery	6 (86)
Radiation therapy	2 (29)
Tyrosine kinase inhibitor [x] [°]	4 (57)

[x] 3 patients received XL184; 1 patient showed an ongoing PR for 14 months before he became progressive, 2 had PD as best result after 12 and 24 weeks. [°] 1 patient had vandetanib for 8 months, followed by 3 months of sunitinib, which was stopped due to side effects.

Table 2 Efficacy analysis

Parameter	
Median duration of treatment (weeks; range)	17 (6-116)
Cumulative dose of everolimus (mg; median, range)	1200 (440-8012)
Median duration of follow-up (weeks; range)	28 (17-147)
Best response by RECIST 1.0 (*n*, %)	
Complete response	0 (0)
Partial response	0 (0)
Stable disease	5 (71)†
Progressive disease	2 (29)
Overall disease control	5 (71)
Median duration of SD (weeks; range)	24 (17-116)‡
Median PFS (weeks; 95% CI)	33 (8-56)
Median OS (weeks; 95% CI)	30 (15-45)

RECIST Response Evaluation Criteria in Solid Tumors, SD stable disease, PFS progression-free survival, OS overall survival.
† 4 of 5 patients showed SD >24 weeks, ‡ at time of data analysis, 1 patient still had ongoing SD.

Figure 1 Kaplan-Meier curves of (A) overall survival and (B) median progression-free survival

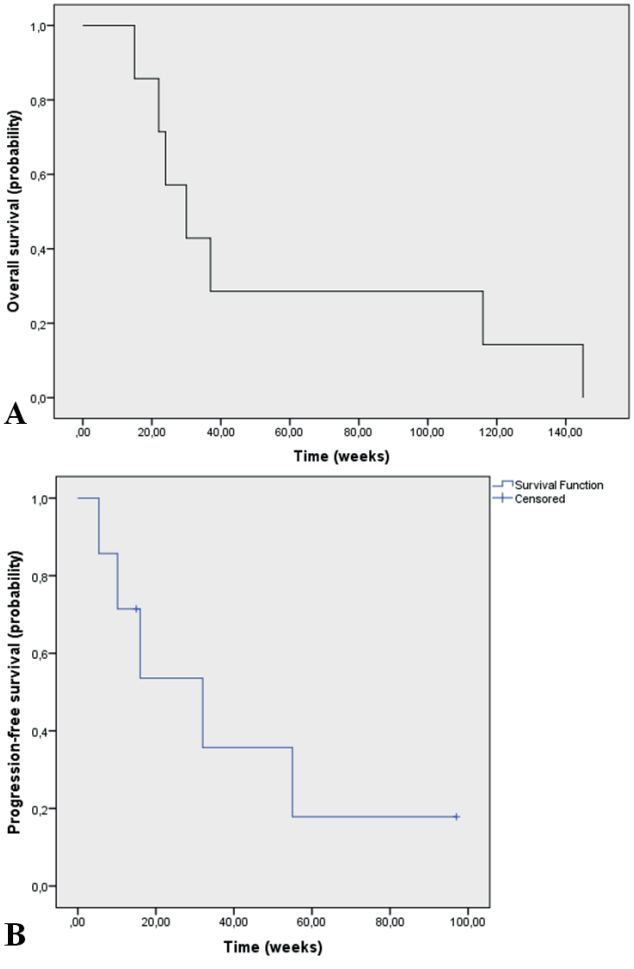
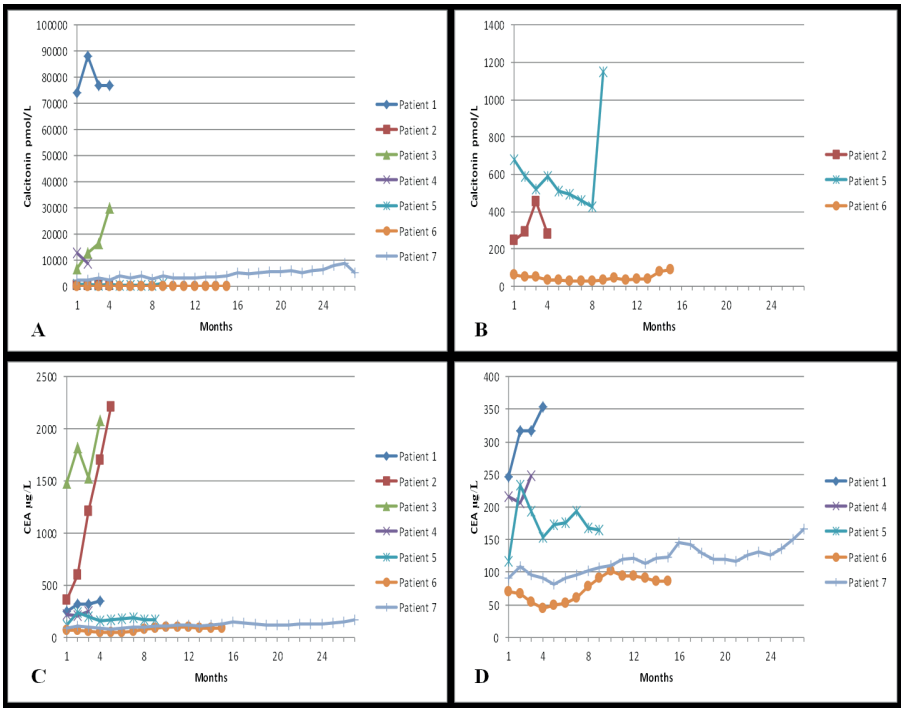


Table 3 Changes in calcitonin and CEA during treatment

Patient		Level at baseline	Maximum reduction (%)	Level at study exit	Increase from baseline till study exit (%)	Best response
1	CT (pmol/L) CEA (µg/L)	73900 246,5	- -	77700 353,3	105,1 143,3	SD
2	CT (pmol/L) CEA (µg/L)	245 354,3	- -	280 2208,0	114,3 623,2	SD
3	CT (pmol/L) CEA (µg/L)	6750 1477,0	- -	29800 2081,0	44,1 140,9	PD
4	CT (pmol/L) CEA (µg/L)	12716 216,9	30,3 4,9	8869 247,9	‡ 114,3	PD
5	CT (pmol/L) CEA (µg/L)	680 117,1	37,3 -	1148 164,4	168,8 114,3	SD
6	CT (pmol/L) CEA (µg/L)	58 70,7	56,9 35,5	89 86,7	153,4 122,6	SD
7	CT (pmol/L) CEA (µg/L)	2156 91,1	- 10,1	5239 ⁺ 166,7 ⁺	243 ⁺ 183 ⁺	SD

CT calcitonin, CEA carcinoembryonic antigen level, - there was no reduction in CT or CEA compared to baseline, ‡ there was no increase in CT compared to baseline ⁺patient is still on everolimus treatment

Figure 2 Serum calcitonin and CEA concentrations per patient over time



A: serum calcitonin over time for all patients, B: close-up of figure A for patients 2, 5 and 6, C: serum CEA for all patients, D: close-up of figure C for patients 1, 4, 5, 6 and 7.

Toxicity

Three patients (43%) required dose reduction due to toxicity. One patient (14%) discontinued the study after 12 weeks due to complaints of fatigue and peripheral edema. All observed AEs are listed in table 4. Treatment-related AEs were predominantly grade 1 or 2, with the most common events including mucositis, fatigue and hypertriglyceridemia. Grade 3 AEs consisted of fatigue (29%), peripheral edema (14%), hypercholesterolemia (14%), hyperglycemia (14%), pneumonia (14%) and pneumonitis (14%). No grade 4 AEs were observed. One (14%) serious adverse event (SAE) was reported when a patient was hospitalized due to acute stomach pain after 5 weeks of everolimus treatment. A gastroduodenoscopy revealed no abnormalities and the complaints resolved spontaneously. The majority of AEs were controllable with dose reduction, medication or supporting measures. There appeared to be no relation between toxicity and performance state or age.

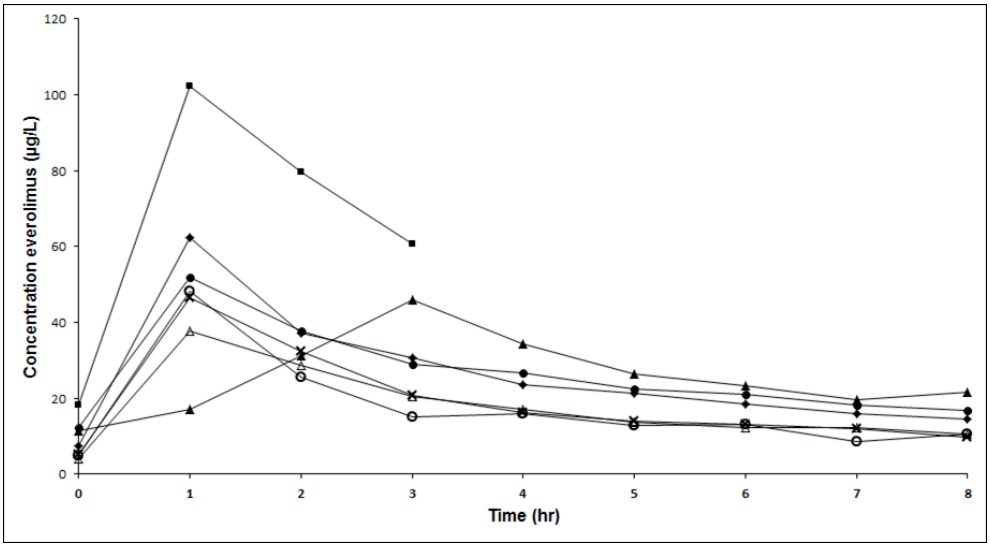
One patient with relatively high everolimus blood plasma concentrations ($AUC_{0\text{-}24hr}$, 960µg*hr/L), as shown in figure 3, also showed more profound toxicity. This patient suffered a total of 13 AEs, whereas the median number of AEs was 5 (range 3-13). Co-medication and a high hematocrit were excluded as possible causes of the higher plasma concentrations in this patient.

Table 4 Adverse events

	All	Grades Number of patients (% of category)			
Event	**Number of patients (% of total (*n*=7))**	**1**	**2**	**3**	**4**
Mucositis	3 (43)	2 (67)	1 (33)		
Fatigue	3 (43)	1 (33)		2 (67)	
Hypertriglyceridemia	3 (43)	1 (33)	2 (67)		
Peripheral edema	2 (29)		1 (50)	1 (50)	
Anorexia	2 (29)	2 (100)			
Diarrhea	2 (29)	1 (50)	1 (50)		
Rash	2 (29)	1 (50)	1 (50)		
Pneumonia	2 (29)	1 (50)		1 (50)	
Liver function #	2 (29)	1 (50)	1 (50)		
Hypercholesterolemia	2 (29)	1 (50)		1 (50)	
Hypophosphatemia	2 (29)	2 (100)			
Hypoparathyroidism	2 (29)	2 (100)			
Weight loss	1 (14)	1 (100)			
Nausea	1 (14)	1 (100)			
Vomiting	1 (14)	1 (100)			
Constipation	1 (14)	1 (100)			
Allergic reaction	1 (14)		1 (100)		
Dry skin	1 (14)	1 (100)			
Itch	1 (14)		1 (100)		
Asthenia	1 (14)	1 (100)			
Anemia	1 (14)		1 (100)		
Hyperglycemia	1 (14)			1 (100)	
Cough	1 (14)		1 (100)		
Dyspnea	1 (14)	1 (100)			
Pneumonitis	1 (14)			1 (100)	

All AEs graded according to Common Terminology Criteria for Adverse Events version 4.0

Figure 3



Individual observed concentration versus time profiles for everolimus

Mutation analysis

One of the 7 MTC patients was excluded from the mutation analysis because no tumor tissue was available. Of the 6 remaining patients, one MEN2A patient carried a germline *RET* c.1858T>C, p.Cys620Arg mutation. Using targeted nextgen sequencing of tumors, four showed a somatic *RET* c.2753T>C, p.Met918Thr mutation, including one that also carried an *EGFR* c.2543C>T, p.Pro848Leu mutation (see supplementary table 1). It is noteworthy that the MEN2A patient showed the best response to everolimus treatment, with the longest period of stable disease.

Pharmacokinetics

One patient completed only the short PK sampling schedule; all other patients participated in the extended sampling schedule up to 8 hours after everolimus intake. Individual everolimus concentrations versus time profiles are shown in figure 3.

A summary of everolimus pharmacokinetics is shown in table 5. The median (range) $AUC_{0\text{-}24hr}$ for everolimus was 421 µg*hr/L (257-960 µg*hr/L) and the median C_{trough} was 7.4µg/L (4.0-18.3 µg/L). The median T_{max} was 1.0 hour and the median C_{max} was 48.2 µg/L. The median $T_{1/2}$ of everolimus was 13.8 hours (10.9-32.4 hr).

Table 5 Summary of everolimus pharmacokinetic parameters

	Median (range)	Mean	SD	CV%
$AUC_{0\text{-}24hr}$ (µg*hr/L)	421 (257-959)	442	246	55.7%
C_{trough} (µg/L)	7.4 (4-13.8)	9.0	5.2	57.8%
T_{max} (hr)	1 (0.33-3.08)	1.2	0.9	75.0%
C_{max} (µg/L)	48.2 (37.8-102.3)	59.4	23.8	40.1%
$T_{1/2}$ (hours)	13.8 (10.9-32.4)	16.5	7.5	45.5%

AUC area under the concentration time curve, C_{max} peak plasma concentration, CV% coefficient of variation, SD standard deviation, T_{max} time to reach peak plasma concentration.

DISCUSSION

Until recently, surgery was accompanied by only limited curative and palliative treatment options for patients with MTC, emphasizing the need for new therapies. Using everolimus, a significant dose-dependent inhibition in cell proliferation was observed in two medullary thyroid cancer cell lines [11]. Everolimus significantly inhibited cell viability in a dose and time-dependent fashion, and diminished phosphorylation of mTOR in a TT thyroid cancer cell line and cultured human MTCs [12]. Two case reports comprising three patients with advanced MTC showed beneficial effects of everolimus [11,13]. Recently, a phase II study in patients treated with everolimus, with advanced thyroid cancer of all histologic subtypes (n=38), reported a partial response (PR) and stable disease (SD) in 5% and 76% of patients, respectively. Median progression-free survival (PFS) was 47 weeks [14].

Our phase II trial on advanced MTC showed promising results, with stable disease in 5 of 7 (71%) patients. The median PFS and OS of 33 and 30 weeks respectively, were also promising. The lack of PRs is easily explained by the fact that these tumors are slowly progressive and PRs were therefore not expected. Due to the fact that only seven patients were included, we were not able to identify parameters significantly influencing response or survival. Although several trials have reported a biochemical CEA and calcitonin response that reflects the radiological response, these changes were not always significant and in our study and we were unable to confirm this correlation [11, 14-16].

Although EGFR overexpression is frequently seen in medullary thyroid cancer, EGFR mutations are rarely described [17]. The *EGFR* P848L mutation we identified is situated close to the L858R mutation, a well-known target in non-small cell lung cancer. However, the P848L mutation has been described in patients with non-small cell lung carcinoma and is thought to be a functionally silent polymorphism, insensitive to gefitinib treatment [18,19].

The MEN2A MTC patient showed the best response upon everolimus therapy.

Apart from the treatment effect, the *RET* c.1858T>C, p.Cys620Arg mutation has been associated with a less aggressive phenotype compared to other MEN2A-related mutations such as the c.2753T>C p.Met918Thr mutation or one of the classic mutations in exon 11 at codon 634 [20]. To the best of our knowledge, this is the first report of administration of everolimus to a MEN2A patient.

Everolimus was generally well tolerated, with the majority of AEs manageable and similar to previously reported toxicities [14,21]. The PK characteristics were also consistent with phase I pharmacokinetic studies investigating everolimus (10 mg once daily) in solid and hematological malignancies [22-24]. The inter-patient variability in PK was also comparable to previous research. Although based on the short sampling schedule, the patient with the highest exposure also showed the most extensive toxicity profile, perhaps related to higher everolimus plasma concentrations.

Although everolimus treatment has shown encouraging results in patients with a variety of solid tumors, it has to be noted that its inability to inhibit mTORC2 may imply an inability to achieve a potent and long term antitumor effect. Hisamatsu et al. showed that simultaneous inhibition of mTORC2 during everolimus treatment enhanced the anti-tumor effect of everolimus and prevented clear cell carcinoma cells of the ovary from acquiring resistance to RAD001 [25]. Furthermore, several reports has been published describing a feedback loop between the mTOR pathway and RAS/MAPK/ERK signaling, leading to activation of a different prosurvival signaling pathway upon mTOR inhibition. This mechanism of action suggests that treatment with everolimus as a single molecular target agent may not be sufficient, emphasizing the interest of studies with everolimus combined with other targeted agents [6,14,26].

In conclusion, given the high rate of clinical benefit and the relatively low toxicity profile found in this MTC subgroup analysis, we believe that further investigation in larger cohorts of MTC patients is now warranted, using everolimus either as a single agent or in sequential or combination therapy.

Supplementary Table 5 Somatic mutations

Sample ID	Gene	Chromosome	Chromo-some position	Exon	Coverage	Mutation Frequency (%)		AA change	Cosmic ID
MTC29	RET	10	43617416	16	85	21,18	c.2753T>C	p.Met918Thr	COSM965
MTC31	RET	10	43617416	16	1751	64,13	c.2753T>C	p.Met918Thr	COSM965
MTC33	RET	10	43617416	16	606	64,13	c.2753T>C	p.Met918Thr	COSM965
MTC34	EGFR	7		21	2093	51,84	c.2543C>T	p.Pro848Leu	COSM22943
MTC34	RET	10	43617416	16	647	50,85	c.2753T>C	p.Met918Thr	COSM965
MTC34	RET	10	43609101	10			c.1858T>C	p.Cys620Arg	COSM29804

The reference sequences used were NM_020975.4 for RET and NM_005228 for EGFR

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5

EVEROLIMUS PHARMACOKINETICS AND ITS EXPOSURE-TOXICITY RELATIONSHIP IN PATIENTS WITH THYROID CANCER

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ABSTRACT

Background

Everolimus is a mTOR inhibitor used for the treatment of different solid malignancies. Many patients treated with the registered fixed 10 mg dose once daily are in need of dose interruptions, reductions or treatment discontinuation due to severe adverse events. This study determined the correlation between systemic everolimus exposure and toxicity. Additionally, the effect of different covariates on everolimus pharmacokinetics (PK) was explored.

Methods

Forty-two patients with advanced thyroid carcinoma were treated with 10 mg everolimus once daily. Serial pharmacokinetic sampling was performed on day 1 and 15. Subsequently, a population PK model was developed using NONMEM to estimate individual PK values used for analysis of an exposure-toxicity relationship. Furthermore, this model was used to investigate the influence of patient characteristics and genetic polymorphisms in genes coding for enzymes relevant in everolimus PK.

Results

Patients who required a dose reduction (n = 18) due to toxicity at any time during treatment had significant higher everolimus exposures (mean $AUC_{0\text{-}24}$ (SD) 600 (274) vs. 395 (129) µg*hr/L, P = 0.008) than patients without a dose reduction (n = 22). A significant association between everolimus exposure and stomatitis was found in the four-level order logistic regression analysis (P = 0.047). The presence of at least one TTT haplotype in the *ABCB1* gene was associated with a 21% decrease in everolimus exposure.

Conclusion

The current study showed that dose reductions and everolimus induced stomatitis were strongly associated with systemic everolimus drug exposure in patients with cancer. Our findings confirm observations from another study in patients with cancer and show us that everolimus is a good candidate for individualized dosing in patients with cancer.

Keywords

Everolimus, Exposure-toxicity, Individualized dosing, Pharmacogenetics, Population pharmacokinetics

INTRODUCTION

Everolimus is an orally administered rapamycin derivative inhibiting the mammalian target of rapamycin (mTOR) [1]. This is a key signaling molecule in the phosphatidylinositol 3-kinase (PI3K)/Akt pathway which is involved in the regulation of growth, proliferation, metabolism, survival and angiogenesis of cells and often dysregulated in cancer [1]. Currently, everolimus is registered for the treatment of advanced hormone receptor positive (HR^+), human epidermal growth factor-2 negative ($HER2^-$) breast cancer in postmenopausal women in combination with exemestane, for metastatic renal cell carcinoma (mRCC), for irresectable or metastatic pancreatic neuroendocrine tumors (pNET) and subependymal giant cell astrocytoma (SEGA) [2-4].

Despite its proven efficacy, everolimus is also associated with a number of serious side effects. Most common toxicities associated with everolimus therapy include stomatitis, rash, fatigue, diarrhea, infections, nausea, loss of appetite, hematologic toxicities, dyspnea, noninfectious pneumonitis and metabolic abnormalities such as hypercholesterolemia and -glycaemia [5]. While it is reported that the majority of these adverse events are manageable and of mild to moderate severity, many patients are in need of dose interruptions, reductions or treatment discontinuation due to toxicity [6]. Indeed, in the pivotal breast cancer, mRCC and pNET phase III trials, 10 to 35% of the patients discontinued everolimus treatment due to adverse events [2-4]. In addition, ~62% of the patients needed dose interruptions or reductions compared to 12-29% in the placebo arms [2,4].

The large number of dose reductions and treatment discontinuation make toxicity currently one of the main challenges in the optimal use of everolimus for the treatment of cancer. In oncology, everolimus is registered as a fixed oral dose of 10 mg once daily. However, in transplantation medicine therapeutic drug monitoring (TDM) with individualized dosing is routinely applied due to everolimus' narrow therapeutic window and high inter-patient variability in pharmacokinetics (PK) [7]. In transplantation medicine, everolimus is used as an immunosuppressant to prevent rejections. Dose individualization is not only applied to prevent toxicity, but also to optimize treatment efficacy. In oncology, the same high inter-patient variability in PK is seen (AUC; 45 CV%, C_{trough}; 60 CV%) [8]. This substantial variability, in combination with the fixed 10 mg dosing, results in large differences in everolimus exposure between patients. This could result in either supra-therapeutic drug exposure with an increased incidence of toxicity, but also in sub-therapeutic drug exposure leading to decreased anticancer effects.

The primary objective of this study was to assess the correlation between everolimus exposure and toxicity in patients with advanced thyroid cancer. Additionally, we explored the influence of different covariates on everolimus PK, including genetic polymorphisms in genes encoding enzymes involved in the absorption and metabolism of everolimus.

MATERIAL AND METHODS

Patients

Forty-two patients were enrolled in this phase II study investigating the efficacy and pharmacokinetics of everolimus for the treatment of progressive or recurrent, unresectable or metastatic thyroid cancer. The efficacy data of this study will be reported separately. Participating medical centers were the Leiden University Medical Center and the University Medical Center Groningen. Patients were treated continuously with everolimus at an once daily oral dose of 10 mg until tumor progression, unacceptable toxicity, death or discontinuation from the study for other reasons. Toxicities were assessed at baseline, day 1, 14 and 28 of therapy and monthly thereafter according to the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (CTC-AE) version 4.0. Dose adjustments were permitted for adverse events suspected to be related to everolimus. The first dose reduction was to 5 mg once daily. If another dose reduction was needed, everolimus was dosed as 5 mg every other day. The study was approved by the institutional ethics committees (Leiden University Medical Center and University Medical Center Groningen, The Netherlands) and all patients gave written informed consent before entering the study.

Pharmacokinetic sample collection and analysis

For everolimus PK assessment, whole blood samples were obtained at day 1 and 15 of therapy. Samples were collected into EDTA-tubes at pre-dose and 1, 2, and 3 hours after everolimus intake (sparse schedule). More extensive PK sampling at 4, 5, 6, 7 and 8 hours after everolimus intake was optional for patients (extensive schedule). Samples were stored at -20 °C until the day of analysis.

Everolimus concentrations in whole blood were measured using a validated Ultra Performance Liquid Chromatography - Tandem Mass Spectrometric (UPLC-MS/MS) assay. Validation of the assay was performed according to the EMA guidelines of bioanalytical method development [9]. The calibration line was linear over the range from 2 to 160 µg/L and the lower limit of quantification (LLOQ) was 0.6 µg/L. Assay performance was in agreement with guidelines for bioanalytical method development and validation.

Pharmacogenetic analysis

Single Nucleotide Polymorphisms and haplotype selection

Everolimus is metabolized by the cytochrome P450 (CYP) enzymes CYP3A4, CYP3A5 and CYP2C8 and is also a substrate for the efflux pump P-glycoprotein (P-gp) encoded by the *ABCB1* gene [10]. The nuclear pregnane X receptor (PXR; NR1I2) regulates the expression of CYP3A4 and could therefore also influence everolimus PK [11]. Eleven

single nucleotide polymorphisms (SNPs) in these genes were selected based upon a candidate gene approach (Supplementary Data S1, online). For the *ABCB1* and *CYP2C8* gene, selected SNPs were used for haplotype analysis performed in gPLINK (Supplementary Data S2, online). Haplotypes were set at a certainty greater than 0.97. For the *ABCB1* and *CYP2C8* gene, only haplotypes and no individual SNPs were tested.

Genotyping assays

Germline DNA was isolated from 400 µl EDTA-blood using MagNa Pure Compact (Roche, Almere, the Netherlands). DNA concentrations were thereafter measured using Nanodrop (Isogen, De Meern, The Netherlands). Genotyping was performed using pre-designed genotyping assays (Supplementary Data S1, online). Samples were analyzed on a Viia7 real-time PCR system according to the manufacturers' instruction (Life Technologies, Bleiswijk, The Netherlands). Call rates of all assays were >98%. As a quality control, at least 5% of the samples were genotyped in duplicate. No inconsistencies were observed. Minor allele frequencies (MAF) of all 11 SNPs were calculated and compared with reported MAF for European Populations (HAPMAP). No significant deviations were observed and derived allele frequencies were all in Hardy-Weinberg equilibrium ($P \geq 0.05$) (Supplementary Data S1, online).

Pharmacokinetic modelling

Base model

Thirty patients completed the extensive PK sampling and ten patients the sparse PK sampling schedule. After PK sampling, nonlinear mixed-effects modeling (NONMEM) was used to describe the population pharmacokinetics of everolimus. Subsequently, the developed population PK model was used to estimate individual everolimus exposure both in terms of $AUC_{0\text{-}24}$ by using clearance as well as with use of by the model predicted C_{trough} levels. NONMEM version 7.2 (Icon Development Solutions, Ellicott City, MD, USA) was used with Piranã (version 2.9.0) as the modelling environment. Statistical software package R (version 2.15.1) was used for handling of data and plots generation. We also used NONMEM to explore the influence of different covariates on everolimus PK.

A first-order conditional estimation method with interaction (FOCE-I) was used to fit models throughout the building process. One- and two-compartment models with first-order elimination were explored. It was also assessed whether there was a change in clearance from day 1 to day 15 of treatment. Model selection was based on goodness of fit and statistical significance. An adjusted model was chosen over the original model if the drop in the objective function value (OFVs) was > 3.84 ($P < 0.05$ with one degree of freedom (df), assuming χ^2-distribution).

Since the bioavailability (F) of everolimus is unknown, F was fixed at 1 and PK parameter estimates reported are proportional to F except K_a. In addition, both clearance (Cl/F) and the volume of distribution (Vd/F) were allometrically scaled [12].

Covariate analysis

After the base model was determined, covariates were tested to explore the influence of bilirubin, aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), creatinine, body surface area (BSA) and haematocrit on Cl/F. Individual effect sizes were estimated with the formula $Cl/F_{typical\ value} = \theta_1 * (COV/COV_{median})^{\theta 2}$, whereby θ_1 is the population estimate for Cl/F, COV the tested covariate and θ_2 the covariant effect size estimate.

The influence of SNPs and haplotypes were all tested as a covariate on Cl/F, except for *ABCB1* haplotypes which were tested for an effect on F as this is physiologically more plausible. Effect sizes were estimated with the formula $Cl/F_{typical\ value}$ (or F) = $\theta_1 * \theta_2^{\wedge pg1} * \theta_3^{\wedge pg2}$, whereby θ_1 is the population Cl/F or F estimate in wild-type patients, θ_2 the covariate effect size of the heterozygote mutation status and θ_3 the effect size of the homozygote mutation status. The heterozygote (pg1) and homozygote (pg2) mutation status was scored as 1 if present or 0 if not present. If the genotype frequency was < 0.1, homozygote mutant and heterozygote mutant genotypes were combined (Supplementary Data S1 and S2, online)

All covariates were first tested for statistical significance with univariate forward inclusion into the base model (drop in OFV > 3.84, df = 1, $P < 0.05$). After inclusion of significant covariates in the intermediate model, a stepwise backward elimination procedure was performed. Covariates were remained in the final model if the threshold for statistical significance of backward elimination was reached (increase in OFV > 6.64, df = 1, $P < 0.01$).

Evaluation of model fit

Next to goodness of fit plots, a visual predictive check (VPC) was used to assess the performance of the final model by comparing the 10th and 90th percentiles of the simulated concentrations with those of the observed concentrations. In addition, a bootstrap analysis was performed to evaluate the precision of parameter estimation. Shrinkage in inter-individual variability and residual errors were automatically calculated by NONMEM.

Assessment of systemic exposure toxicity relationship

Selection of toxicities

In this study, all experienced toxicities were scored according to CTC-AE version 4.0. However, due to the number of patients included, only a limited number of toxicities were selected to be tested for an association with everolimus exposure in order to prevent false positive findings.

We choose dose reductions as the first outcome of toxicity as this is the sum of all different toxicities experienced by patients and these are also the toxicities that lead to clinical

action by the treating physician. In addition, we selected stomatitis and pneumonitis as toxicity outcomes. The rationale for selection of these toxicities was based on their prevalence and the fact that these toxicities are 1) objectively measurable, 2) clinically relevant and 3) untreatable and therefore leading to dose reductions or discontinuation of therapy. Toxicities were scored as the highest grade experienced until dose reduction and if no reduction occurred until the end of study.

Statistical analysis

The difference in day 15 steady-state everolimus exposure (AUC_{0-24} and C_{trough}) between patients with and without dose reductions was tested with an unpaired t-test. The relationships between day 15 everolimus exposure (AUC_{0-24} and C_{trough}) and stomatitis and pneumonitis were evaluated using a four-level ordered logistic regression in SPSS version 20.0 (IBM).

RESULTS

Patient characteristics

Forty-two adult patients with thyroid carcinoma, 22 men and 20 women were included in the phase II trial that investigated everolimus for the treatment of thyroid cancer. Of these patients, 28 (66.7%) had differentiated, 7 (16.7%) had undifferentiated (anaplastic) and 7 (16.7%) had medullary advanced thyroid carcinoma. Two patients were excluded for PK analysis; in one patient no PK samples were collected, and in the other patient no measurable everolimus levels could be detected. Patient baseline characteristics are shown in Table 1.

Table 1. Patient baseline characteristics

Characteristic	
N	40
Age (years)	63 (40 - 80)
Gender (n)	
Male	21 (52.5%)
Female	19 (47.5%)
Length (cm)	173 (154 - 189)
Weight (kg)	75 (45 - 105)
Hematology	
WBC ($\times 10^9$/L)	7.1 (3.6 - 25)
ANC ($\times 10^9$/L)	4.8 (2.7 - 13.0)
Platelets ($\times 10^9$/L)	254 (147 - 995)
Hemoglobin (mmol/L)	7.5 (5.3 - 10.7)
Haematocrit	0.39 (0.29 - 0.50)
Chemistry	
AST (U/L)	22 (12 - 61)
ALT (U/L)	22 (7 - 19)
Creatinine (µmol/L)	66 (42 - 205)
Total bilirubin (µmol/L)	9 (4 - 16)
Tumor type (n)	
Differentiated	26 (65%)
Undifferentiated	7 (17.5%)
Medullary	7 (17.5%)

Data are presented as median (range) unless stated otherwise. Abbreviations: ALT, alanine aminotransferase; ANC, absolute neutrophil count; AST, aspartate aminotransferase; WBC, white blood count.

Pharmacokinetics

A total of 669 samples from 40 patients were used to build the population PK model. The pharmacokinetic data for everolimus were best described by a two-compartmental model with first order absorption and first order elimination from the central compartment (Supplementary Data S3, online). No difference in clearance over time between day 1 and day 15 of treatment was found.

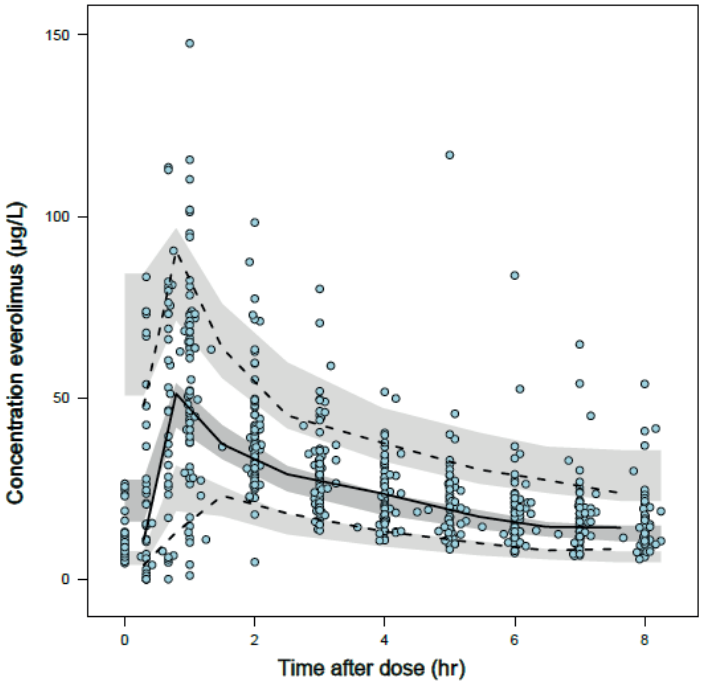
Forward inclusion of BSA, creatinine, ASAT, ALAT, bilirubin and haematocrit did not improve the PK model and no association between these covariates and clearance was found. With forward inclusion of the *ABCB1* TTT and CCG haplotype the base model significantly improved (ΔOFV = -7.2 and -6.4 respectively, P <0.05). The other SNPs and the *CYP2C8* haplotype did not improve the model. With multivariate backward elimination, only the presence of at least one *ABCB1* TTT haplotype remained significant (ΔOFV = 9.6, P <0.01). A 21% decrease in F was observed in the presence of at least one *ABCB1* TTT haplotype. Inclusion of this covariate in the final PK model, reduced the inter-patient variability in Cl/F from 38.1 to 35.1 CV%. Parameter estimates of the base and final model are shown in Table 2.

Evaluation of the final model was, next to inspection of the goodness of fit plots, done with VPC and a bootstrap procedure. Results of the VPC show that predicted and observed concentration intervals are almost identical, indicating accuracy and good predictive performance of the final model (Figure 1). There is a small tendency for a difference between predicted and observed concentrations in the absorption part of the curve due to limited number of samples during this phase. Since we mainly used the model to estimate individual values for Cl/F, the modest under-prediction of the absorption did not affect our analysis. The successful bootstrap procedure with 1000 runs is shown in Table 2. The median values for PK parameters found were within 10% of those estimated with the final model indicating that the model is precise and reliable in its parameter estimation.

Table 2. Summary of model parameter estimates

Parameter	Base model			Final model			Bootstrap runs	
	Estimate	RSE (%)	Shrinkage (%)	Estimate	RSE (%)	Shrinkage (%)	Median value	95-CI
Cl/F (L/hr)	20.3	7.0		17.4	8.4		18.0	15.5 - 20.8
F	1	-		1	-		1	-
V_1/F (L)	29.1	18.5		25.2	17.8		25.7	18.1 - 40.4
k_a (hr^{-1})	0.643	5.3		0.647	6.2		0.653	0.583 - 0.740
Q (L/hr)	60	4.7		51.1	7.3		52.1	45.5 - 59.1
V_2 (L)	475	5.4		400	-		400	-
θ_{TTT} on F	NA	NA		0.792	6.5%		0.81	0.71 - 0.90
Inter-individual variablity								
Cl/F (CV%)	38.1%	34.4	10	35.1%	30.5	11	35.0%	22.1 - 49.1%
V_1/F (CV%)	87.3%	35.7	27	86.4%	35.3	27	90.5%	53.7 - 138.9%
Inter-occasion variability								
F (CV%)	20.7%	37.7	9	19.2%	38.1	12	19.4%	12.9 - 30.5%
Residual variability								
σ (proportional error	27.2%	20.7	7	27.3%	20.8	7	27.9%	22.6 - 32.9%

Figure 1. Visual predictive check (VPC) of final everolimus PK model



Exposure-toxicity relationship

The relationships between everolimus exposure and dose reductions as well as stomatitis and pneumonitis were examined. In total, 45% of the patients had their everolimus 10 mg dose reduced to a lower dose due to toxicity (Table 3). In general, toxicity developed within 3 months after the start of everolimus therapy. Toxicities leading to dose reduction included stomatitis, pneumonitis, fatigue, loss of appetite, diarrhea, liver and kidney toxicity and oedema. Considering stomatitis, 42.5% of the patients experienced any grade stomatitis and 7.5% experienced grade 3 stomatitis. In addition, 10% of the patients had a non-infectious pneumonitis.

Table 3. Dose reductions and toxicity incidence	
Dose reductions	
No	22 (55%)
Yes	18 (45%)
Stomatitis	
None	23 (57.5%)
Grade 1	12 (30%)
Grade 2	2 (5%)
Grade 3	3 (7.5%)
Pneumonitis	
None	36 (90%)
Grade 1	2 (5%)
Grade 2	1 (2.5%)
Grade 3	1 (2.5%)
Reason for reduction	
Stomatitis	4 (22.2%)
Pneumonitis	4 (22.2%)
Fatigue	5 (27.8%)
Loss of appetite	1 (5.6%)
Diarrhea	1 (5.6%)
Liver toxicity	1 (5.6%)
Kidney toxicity	1 (5.6%)
Edema	1 (5.6%)

Figure 2 shows boxplots of everolimus AUC and C_{trough} in patients with and without dose reduction. Mean AUC_{0-24}(SD) and C_{trough} were 600(274) and 395(129) µg*hr/L and 14.9(9.0) and 8.4(3.8) µg/L for patients with and without dose reductions respectively. The exposure to everolimus was significantly different between the two groups (mean difference in AUC -204 µg*hr/L (95%-CI; -340 to -69 µg*hr/L, P = 0.008 and mean difference in C_{trough} -6.5 µg/L (95%-CI; -11.2 to -1.8 P = 0.009). Figure 3 shows boxplots of AUCs and C_{trough} in patients experiencing different grades of stomatitis. A positive association between everolimus exposure and stomatitis was identified (P = 0.047). The odd ratio for stomatitis was 1.16 (95%-CI; 1.06 to 1.26) for every 50 µg*hr/L increase in AUC_{0-24}. Patients with grade 3 stomatitis had an everolimus exposure that was two times that of patients with ≤ 2 stomatitis (AUC_{0-24} 896 vs. 456 µg*hr/L, P > 0.05 and C_{trough} 24.9 vs 10.3 µg/L, P > 0.05). No association of everolimus exposure with pneumonitis was found.

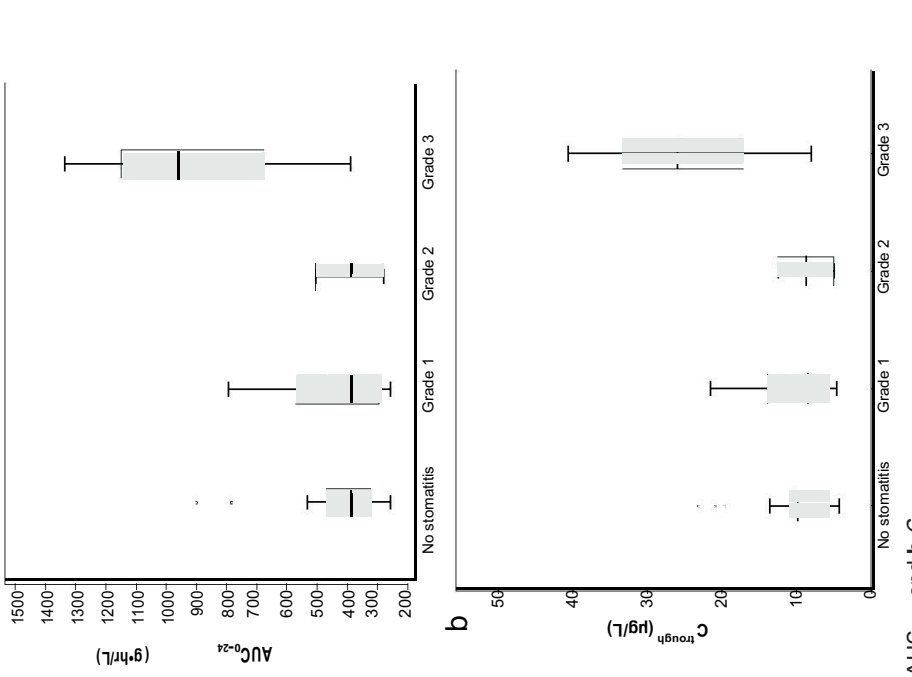


Fig. 3 *Boxplot* of severity of stomatitis versus everolimus exposure. **a** AUC_{0-24} and **b** C_{trough}

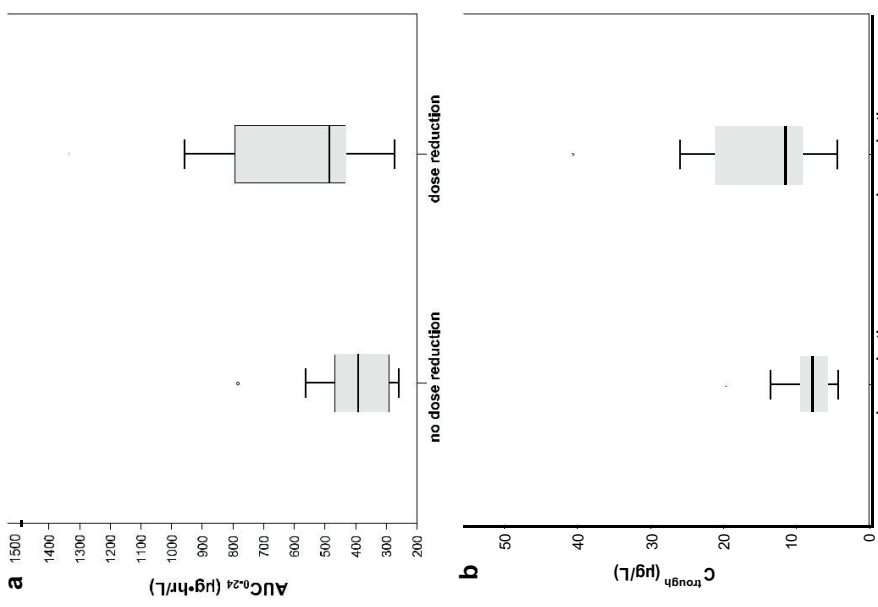


Fig. 2 *Boxplot* of everolimus exposure in patient with and without dose reduction. **a** AUC_{0-24} and **b** C_{trough}

DISCUSSION

This study was primarily performed to assess the correlation between everolimus exposure and toxicity. Results show that patients who had their everolimus dose reduced due to toxicity, had significantly higher drug exposures than patients without the need for dose reductions. Moreover, everolimus exposure was associated with the probability for stomatitis and patients with grade 3 stomatitis had an everolimus exposure two times that of patients with ≤ 2 stomatitis. Additionally, we found that the presence of at least one TTT allele in the *ABCB1* gene was associated with lower everolimus exposure due to decreased absorption.

The present findings of a clear relationship between everolimus exposure and dose reductions due to toxicity and stomatitis are in line with results from other studies in patients with cancer treated with everolimus. Previously, it has been shown that a 2-fold increase in everolimus exposure increased the risk of ≥ grade 3 pulmonary events, ≥ grade 3 stomatitis and ≥ grade 3 metabolic events with 1.9, 1.5 and 1.3-fold respectively in patients with advanced solid tumors [13]. The present analysis could not confirm the earlier identified association of everolimus exposure with pneumonitis, but this may be due to the limited number of patients with pneumonitis in our study cohort.

The fixed 10 mg dosing regimen of everolimus is based on its safety profile together with its pharmacodynamic effects on the mTOR dependent pathway in tumor and skin biopsies [14,15]. These studies suggested a dose of ≥5 mg or ≥10 mg daily, based on complete inhibition of serine/threonine kinase p70S6 kinase (S6K1) or phosphorylated eIF-4G (peIF-4G) which are both downstream targets of mTOR. Since inhibition of peIF-4G was only complete at the 10 mg dose level, it was advised to use the 10 mg once daily schedule for future clinical studies. The clinical relevance of this difference in inhibition of eIF-4G is however unknown and should be further investigated as higher dosing introduces also more toxicity.

The present study underscores the high inter-patient variability in everolimus PK which is in line with previous observations [8]. This is also analogue to the variability in PK seen for other oral targeted therapies for the treatment of cancer such as tyrosine kinase inhibitors (TKIs). For TKIs the evidence for relationships between systemic drug exposure and efficacy or toxicity endpoints is growing [16,17]. The currently available data suggest that an individualized dosing approach seems justified in certain circumstances and different studies support the feasibility of an individualized dosing approach for TKIs [18,19].

In the exploration of covariates of influence on everolimus PK, the presence of at least one TTT haplotype was responsible for a decrease in everolimus exposure due to decreased absorption. Previously, the TTT haplotype has been demonstrated to be associated with enhanced function of the P-glycoprotein transporter and indeed

reduced exposure or efficacy of treatment [20-22]. However, decreased function of the transporter and thus increased exposure have also been reported, as well as studies that could not show an effect [7,23,24]. The association we found should be regarded as preliminary and needs further validation. If this association is confirmed, it might be argued whether a decrease in exposure of 21% can be considered as clinically relevant when taking into account the inter-patient variability in everolimus PK.

To the best of our knowledge, we are the first to describe the population pharmacokinetics of everolimus 10 mg once daily in patient with cancer. Previously, population PK models have been described, but only within the field of transplantation medicine where everolimus is used in a much lower dose. Taking this and differences in modeling into account, pharmacokinetic parameter estimates were in agreement with those previously found [7].

Everolimus exposures were assessed at day 15 of therapy and not necessarily at the time when adverse events occurred. This may be considered as a limitation and future studies should preferably measure everolimus exposure at the time that toxicity occurs. However, the variability in everolimus PK within a patient (intra-patient) is reported to be much smaller than the variability between patients [25,26]. In addition, we observed a constant clearance of everolimus over time. While treated at the same dose (10 mg once daily), this restricts the probability for large differences between the exposures that we have measured and the actual exposures that would have been measured at the moment that toxicity occurred. In addition, the study that previously described a correlation between everolimus exposure and toxicity, found similar results with the use of C_{trough} at the time of toxicity or when C_{trough} averaged over a given time period was used [13].

The present results both underscore the correlation between everolimus exposure and dose reductions due to toxicity as well as the high inter-patient variability in everolimus PK. These observations should be taken into account in the use of everolimus for the treatment of solid tumors. Preventing high drug exposures by dose individualization may have the potential to reduce the side effects of everolimus therapy while remaining its efficacy. However, prospective validation within oncology patients in necessary. Moreover, it has been shown that high early everolimus exposure (C_{trough} >14.1 µg/L) is associated with longer progression free survival (PFS) and overall survival (OS) (13.3 and 26.2 months vs. 3.9 and 9.9 months for PFS and OS respectively) in patients with mRCC [27]. Hence, an individualized dosing approach may also be of value for some patients with treatment inefficacy due to subtherapeutic exposures. On the other hand, in this present analysis there were also patients in need of dose reductions in whom the exposure to everolimus was not elevated. This finding suggest that a subpopulation may not benefit from dose individualization but maybe more from treatment switch if available. In summary, future studies are required to define the therapeutic window of everolimus for the treatment of different malignancies and these studies should

aim to optimize both treatment toxicity as well as efficacy outcomes possibly by using everolimus in a more individualized way.

CONCLUSION

In conclusion, this study shows a clear association between everolimus exposure and dose reductions due to toxicity as well as stomatitis in patients with cancer using a newly developed population PK model. Our findings confirm observations from another study in patients with cancer and show us that everolimus is a good candidate for individualized dosing in patients with cancer.

Supplementary data 1. Selected polymorphisms in genes involved in the absorption and metabolism of everolimus

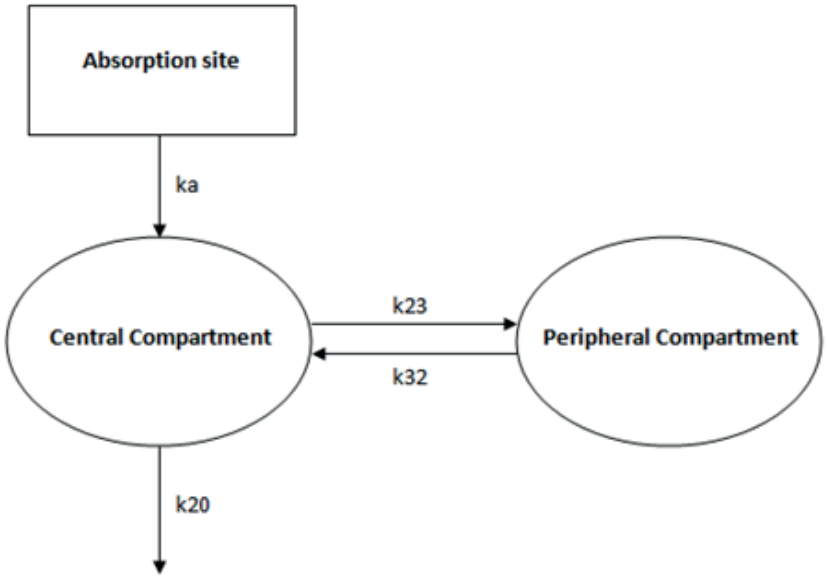
Gene	rs number	Polymorphism	Genotype	Frequency N (%)		Observed Minor Allele Frequency (%)	HWE (*p*-value)	Assay ID	Covariate testing
ABCB1	rs1128503	1236C>T	CC (wt) TC TT	12 25 4	(29.2) (61.0) (9.8)	T = 40.2%	0.09	C___7586662_10	in haploblock
ABCB1	rs2032582	2677G>T/A	GG (wt) GT TT	12 23 5	(30.0) (57.5) (12.5)	T = 41.3%	0.24	C_11711720C_30	in haploblock
ABCB1	rs1045642	3435T>C	TT (wt) TC CC	10 24 7	(24.4) (58.5) (17.1)	C = 46.3%	0.26	C___7586657_20	in haploblock
NR1I2	rs2276707	8055C>T	CC (wt) CT TT	30 8 3	(73.2) (19.5) (7.3)	T = 17.1%	0.05	C__15882324_10	CC vs. CT+TT
NR1I2	rs6785049	7635A>G	AA (wt) AG GG	15 19 7	(36.6) (46.3) (17.1)	G = 40.2%	0.82	C__29280426_10	AA vs. AG vs. GG
CYP3A5	rs776746	6986A>G	GG/*3*3(wt) AG/*1*3 AA/*1*1	32 7 2	(78.0) (17.1) (4.9)	A = 13.4%	0.09	C__26201809_30	GG vs. AG+AA
CYP3A4	rs2246709	16090A>G	AA (wt) AG GG	19 15 6	(47.5) (37.5) (15.0)	G = 33.8%	0.31	C___1845287_10	AA vs. AG vs. GG
CYP2C8	rs7909236	-271G>T	GG (wt) GT TT	21 19 1	(51.2) (46.4) (2.4)	T = 25.6%	0.17	custom designed *	GG vs. GT+TT
CYP2C8	rs10509681	47603213T>C	TT (wt) CT	36 5	(87.8) (12.2)	C = 6.1%	0.68	C__25625782_20	in haploblock
CYP2C8	rs11572080	47631494C>T	CC(wt) TC	36 5	(87.8) (12.2)	T = 6.1%	0.68	C__25625794_10	in haploblock
CYP3A4	rs35599367	522-191C>T	CC (wt) CT	39 2	(95.1) (4.9)	T = 4.9%	0.87	C_59013445_10	not tested due to too low frequency

* custom designed assay: PCR primers, forward: 5′-GTATTGGATTGGAGCCCAGGTATTT-3′, reverse: 5′-TGTTTCTCCATCATCACAGCACAT-3′; probes, VIC: AAGTCCCTGGTTGTTCCA, FAM: TCCCTGGTTTTTCCA

Supplementary data 2. Selected haploblocks in genes involved in the absorption and metabolism of everolimus

Gene	rs number	Polymorphism	Genotype	Frequency N (%)	Observed Minor Allele Frequency (%)	Covariate testing
ABCB1 haploblock	rs1128503 rs2032582 rs1045642	1236C>T 2677G>T/A 3435T>C	other-other TTT-other TTT-TTT	23 (56.1) 14 (34.1) 4 (9.8)	TTT= 26.8%	other-other vs. TTT-other + TTT-TTT
			other-other CTG-other CTG-CTG	30 (73.2) 10 (24.4) 1 2.4)	CTG = 14.6%	other-other vs. CTG-other + CTG-CTG
			other-other CCG-other CCG-CCG	10 (24.4) 26 (63.4) 5 (12.2)	CCG = 43.9	other-other vs. CCG-other vs. CCG-CCG
CYP2C8 haploblock	rs10509681 rs11572080	47603213T>C 47631494C>T	CT-CT TC-CT	36 (87.8) 5 (12.2)	TC = 6.1%	CT-CT vs. TC-CT

Supplementary data 3. Schematic presentation of PK model



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6

EVEROLIMUS IN PATIENTS WITH ADVANCED FOLLICULAR-DERIVED THYROID CANCER; RESULTS OF A PHASE II CLINICAL TRIAL

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The medication used in this trial was provided by Novartis. Furthermore, Novartis financially supported the conduction of this trial

ABSTRACT

Background

mTOR upregulation has been reported to be involved in the pathogenesis of thyroid tumors and treatment with the mTOR inhibitor everolimus has shown promising results in endocrine tumors. We conducted a prospective phase II clinical trial to determine the efficacy and safety of everolimus in patients with advanced follicular-derived thyroid cancer.

Patients and methods

Twenty-eight patients with progressive metastatic or locally advanced radioactive refractory differentiated thyroid cancer and 7 patients with anaplastic thyroid cancer were included and received everolimus 10mg orally once daily. The primary endpoint was disease control rate (complete (CR) + partial response (PR) + stable disease (SD) >24 weeks). Secondary endpoints included progression free survival (PFS), overall survival (OS), toxicity and mutational and pharmacokinetic related outcomes (PK).

Results

Median follow-up duration was 38 months (2-64). Seventeen patients (65%) showed SD, of which 15 (58%) showed SD >24 weeks. No CR or PR were observed. Median PFS and OS were 9 (95%CI:4-14) and 18 (95%CI:7-29) months, respectively. Survival was negatively influenced by the presence of bone metastases. Toxicity was predominantly grade 1/2 and included anemia (64%), cough (64%), stomatitis (61%) and hyperglycemia (61%). Duration of SD was related to everolimus exposure. The presence of somatic gene variants related to mTOR signaling did not clearly stratify for responses.

Conclusion Everolimus has clinically relevant antitumor activity in patients with advanced differentiated thyroid cancer. Given the observed disease control rate and the relatively low toxicity profile, further investigation of everolimus in sequential or combination therapy in these patients is warranted.

Key terms

Everolimus, differentiated thyroid cancer, anaplastic thyroid cancer, phase II, clinical trial

INTRODUCTION

Thyroid cancer is the most prevalent endocrine malignancy, accounting for 96% of cancers of the endocrine system and 66% of endocrine cancer mortality in 2012 (1). The current incidence in Europe is 49/1,000,000 per year, with a nearly three times higher incidence in women (2). Thyroid cancer is a heterogeneous disease that is classified into differentiated thyroid carcinoma (DTC), undifferentiated (anaplastic) thyroid carcinoma (ATC) and medullary thyroid carcinoma (MTC). Differentiated thyroid carcinoma (DTC) is by far the most common (95%) subtype, and includes papillary (PTC, 80%) and follicular (FTC, 10-15%) as well as subtypes like oncocytic thyroid carcinomas (FTC-OV). The majority of DTCs are slowly progressive, and, when identified at an early stage, frequently cured with surgical management and radioactive iodine 131-I ablation (RAI) therapy. However, metastatic DTC that has become inoperable or refractory to RAI therapy, is associated with a less favorable prognosis, as 10-year survival then varies between 25% to 40% (3,4). The efficacy of conventional chemotherapy in DTC is negligible, and chemotherapy is therefore no longer recommended in international guidelines (5,6). In fact, the American Thyroid Association recommends enrolling these patients in clinical trials. As a result of increased understanding of thyroid tumorigenesis, potential targets and novel therapeutic agents that target biological abnormalities have been identified. Until now, sorafenib and lenvatinib are the only targeted agents registered for the treatment of advanced DTC. Despite the improvement in PFS in a proportion of patients with these compounds, no effect on survival has been observed. Therefore, additional treatment options are needed for patients with progressive DTC.

Recently, several genetic alterations involving tyrosine kinase signaling pathways have been described in the pathogenesis of thyroid cancer. Activating *RAS*, *BRAF* and *TERT* somatic gene variants, as well as *RET/PTC* rearrangements, lead to the constitutive activation of the MAP kinase pathway which in turn plays a key role in the process of tumor development and progression (7-9). In addition to the *RET-RAS-RAF-MAPK* signaling pathway, the (PI3K)/Akt pathway is involved in the regulation of cell growth, proliferation and survival (10). An important down-stream effector in the PI3K/Akt pathway is the mammalian target of rapamycin (mTOR). Activation of the mTOR serine/threonine protein kinase has been reported in a variety of malignant tumors with an estimated 70% of mTOR upregulation in all tumors (11). Furthermore, mTOR was demonstrated to be activated in aggressive PTC (12). Several studies report genomic alterations with activating effect on mTOR signaling including *MTOR*, *TSC1*, *TSC2*, *NF1, PTEN* and *PIK3CA* to induce sensitivity to mTOR inhibitors (13,14).

As a result of this accrued understanding of the biological basis for (differentiated) thyroid cancer development, multiple clinical trials with multi-target tyrosine kinase inhibitors (TKIs) have been conducted (15). Everolimus (RAD001) is

an orally available derivative of rapamycin, targeting mTOR. Everolimus exerts its activity through high affinity interaction with an intracellular receptor protein, the immunophilin FKBP12. The FKBP12/RAD001 complex subsequently interacts with the mTOR protein kinase, inhibiting downstream signalling events involved in regulation of the G1 to S-phase transition (16). The use of everolimus in neuroendocrine tumors has shown encouraging results, both in vitro and in vivo (17-19). Recently, a phase II study of everolimus in patients with advanced thyroid cancer of all histologic subtypes (n=38), that started simultaneously with our study, reported a partial response (PR) and durable stable disease (SD) in 5% and 45% of patients, respectively. The median progression free survival (PFS) was 47 weeks (20). Another study of everolimus in DTC (n=31) showed one (3%) patient with a PR and 18 (58%) with durable SD. PFS was 16 months and one year survival was 76% (21). Wagle et al showed one patient diagnosed with ATC derived from FTC-OV with striking response upon giving everolimus due to a homozygous somatic *TSC1* variant. This patient showed relapse when the tumor was selected for a secondary somatic *MTOR* variant (22).

Our phase II study was designed to assess the efficacy and safety of everolimus in patients with progressive unresectable recurrent or metastatic differentiated thyroid carcinoma.

MATERIALS AND METHODS

Patients

Eligibility criteria were the presence of per RECIST 1.1 (Response Evaluation Criteria In Solid Tumors) documented progressive metastases or unresectable local recurrence of DTC within 12 months before therapy for which RAI therapy was no longer effective, as indicated by prior negative post-therapeutic RAI scintigraphy (23). Patients were required to have undergone total thyroidectomy and RAI ablative therapy and had to be ≥ 18 years of age with a Karnofsky performance score > 70%. Laboratory requirements consisted of adequate bone marrow function (absolute neutrophil count ≥ 1,500/μL, platelets ≥100,000/μL, hemoglobin ≥ 5.6mm/L), liver function (serum bilirubin ≤ 1.5× upper limit of normal (ULN), serum ALT and AST ≤ 2.5× ULN) and renal function (serum creatinine ≤ 2× ULN). Women of childbearing potential were required to have a negative serum or urinary pregnancy test within 14 days prior to the first dose of study drug. Patients were excluded when they had received prior targeted therapy with everolimus or other mTOR inhibitors. However, prior therapy with other kinase inhibitors was allowed.

Written informed consent was provided by all patients before enrollment in the trial. The study protocol was approved by the Institutional Review Board of the Leiden University Medical Center and performed in the university hospitals of Leiden

and Groningen. This study was registered at Clinical-Trials.gov (#NCT01118065).

Study design

We performed a non-randomized, open-label, multi-center, single arm phase II trial of everolimus in 28 patients with advanced DTC. The primary objective was to determine the efficacy (response rate and stable disease >24 weeks) of everolimus. Secondary objectives were determination of the maximum percentage of tumor reduction for target lesions, describing activity time to event endpoints (PFS and OS) and assessment of toxicity, adverse events (AEs) and relation between pharmacokinetics and response rate and stable disease > 24 weeks. Eligibility assessments, including a review of medical history and prior treatments, physical examination, and disease staging assessments were performed within 4 weeks prior to the first dose of everolimus. Baseline evaluations, comprising performance status, vital signs and laboratory tests were assessed within 2 weeks prior to initiation of therapy. Everolimus was administered at a dose of 10 mg orally once a day until disease progression, unacceptable toxicity, death, or patients' own request. Objective tumor response and time of progressions was measured according to the RECIST criteria version 1.1 every 12 weeks (± 2 weeks) during the first year, thereafter every 3 months and at study discontinuation.

Additionally, patients with progressive metastases or inoperable recurrent disease of undifferentiated (anaplastic, n=7) or medullary (n=7) thyroid cancer were included. The same inclusion and exclusion criteria were used, apart from the criteria on RAI therapy, since this is not applicable for undifferentiated or medullary thyroid cancer. Results on the MTC patients were published separately (24).

Safety assessments were made every 4 weeks by adverse event collection, standard clinical and laboratory tests, and physical examinations. In case of toxicity or AEs requiring dose adjustments, drug dosing was interrupted or modified according to the protocol. The first dose reduction was to 5 mg once daily. If another dose reduction was needed, everolimus was dosed as 5 mg every other day. AE monitoring was continued for at least 4 weeks following the last dose of study treatment. The incidence, grade and casual relationship of adverse events were graded with the use of Common Terminology Criteria for Adverse Events (CTCAE, version 4.0). After study discontinuation, all patients were followed for survival for 4 weeks.

Laboratory parameters

Serum thyroid stimulating hormone (TSH), free thyroxine (T4), thyroglobulin (Tg), Tg antibody levels and safety parameters were assessed at all visits. Safety parameters included a total blood count as well as serum levels of sodium, potassium and creatinin, lipids, coagulation and renal and liver function.

Somatic gene variant spectrum screening

Somatic hotspot gene variants were analyzed using a custom Ampliseq Cancer Hotspot Panel version 2 that targets 50 cancer related genes. Furthermore a dedicated NGS panel was tested targeting *CDKN1A, CDKN1B, CDKN1C, CDKN2A, CDKN2B, CDKN2C, CDKN2D, NF1* and *TSC1*. Ion Torrent Ampliseq sequencing chemistry (Life Technologies, Foster City, CA was used. Libraries were prepared with 10 ng genomic DNA and each sample was uniquely barcoded using the IonXpress barcodes (Life Technologies). PGM 318 or proton P1 chips were prepared using the Ion Chef system and sequencing was performed at the Personal Genome Machine or the Proton, respectively (Life Technologies). Gene variants were analyzed in the Geneticist Assistant NGS Interpretative Workbench (Softgenetics, version 1.1.8). Variants were classified in 5 classes; only class 4 (Likely Pathogenic) and class 5 (Pathogenic) variants were reported.

Pharmacokinetics

To measure everolimus whole blood concentration levels at steady-state pharmacokinetics (PK), patients were admitted to the hospital for PK sampling on day 15 of treatment. Samples were collected into EDTA-tubes at pre-dose, 1, 2, and 3 hours after everolimus intake. Additional PK sampling at 4, 5, 6, 7 and 8 hours after everolimus intake was optional for patients. Everolimus concentrations in whole blood were measured using a validated Ultra Performance Liquid Chromatography - Tandem Mass Spectrometric (UPLC-MS/MS) assay. At steady-state pharmacokinetics, Area Under the Concentration-time curve over the dosing interval ($AUC_{0\text{-}24hr}$), trough everolimus concentration (C_{trough}), time to reach peak concentration (T_{max}), and peak concentration (C_{max}) were determined using non-compartmental analyses in WinNonlin/Phoenix version 6.3 (Pharsight Corporation, St. Louis, MO).

Statistical analysis

Sample size was based on Fleming's one-stage design, using a significance level (one-sided) of 10% and 90% power (25). A response rate at 24 weeks of less than 5% (P0) would not be sufficient to warrant further investigation. A response rate of 20% (P1) or more would certainly implicate further investigation of everolimus in a phase III setting. This required a total of 28 differentiated thyroid cancer patients in our study (α=0.10, β=0.10, P=5%, P1=20%). The anaplastic thyroid cancer patients were analysed in an explorative cohort in this study.

Endpoints are reported as median (range) or proportions. Estimates of PFS (time from starting study drug to progression or death, whichever occurred first) and OS (time from starting study drug to the date of death of any cause) with associated 95% CIs were obtained using the Kaplan-Meier method. Patients that were progression free and/or alive at the time of data analysis were censored. Variables influencing everolimus

response were analyzed with binominal logistic regression. In order to determine the relation between everolimus exposure and efficacy, the patients were divided in two groups based on exposure above or below median everolimus exposure. Calculations were made using binominal logistic regression. The calculations were performed using SPSS 22.0 for Windows (SPSS, Chicago, IL, USA).

RESULTS

Anaplastic thyroid cancer

Since only 7 patients with ATC were included, no definite conclusions could be drawn. Results of this study in patients with ATC were disappointing, with none of the patients benefiting from treatment. Results on ATC patients are presented separately in supplementary Table 1.

DIFFERENTIATED THYROID CANCER

Patient characteristics

A total of 28 DTC patients were included in this study investigating the effect of everolimus in patients with progressive radioiodine refractory unresectable recurrent or metastatic thyroid carcinoma. The study started May 2010 and follow-up of the study ended on 1 January 2016. Median follow-up period was 38 months (range 2-64 months) and median everolimus treatment period was 11 months (range 1-38 months).

Baseline characteristics are listed in Table 1. Thirteen females (46%) and 15 males (54%) were included with a median age of 64 years (range 40-75). The most common histological subtype was FTC-OV (28%), followed by FTC (25%), PTC (21%), poorly differentiated PTC (11%) and follicular variant PTC (7%). After revision by our pathologist, 1 patient (4%) turned out to have an ATC. At study entry, all patients had metastatic disease. Fifteen patients (54%) were previously treated with the tyrosine kinase inhibitor sorafenib.

Table 1 Baseline characteristics

	All patients (*n*=28)
Gender (*n*, %)	
Female	13 (46)
Male	15 (54)
Age (year; median, range)	64 (40-75)
Time from diagnosis (year; median, range)	3.1 (0.4-15.7)
Histology (*n*, %)	
PTC	6 (21)
FTC	7 (25)
FVPTC	3 (11)
FTC-OV	8 (28)
PDPTC	3 (11)
ATC	1 (4)
Initial TNM stage (*n*, %)	
I (T1 N0 M0)	4 (14)
II (T2 N0 M0)	2 (7)
III (T3 N0 M0)	5 (18)
(T1-3 N1a M0)	2 (7)
IVA (T1-3 N1a M0)	1 (4)
(T4a N0-1 M0)	5 (18)
IVB (T4b any N M0)	3 (11)
IVC (any T any N M1)	6 (21)
Tumor extent at study entry (*n*, %)	
Locally advanced	0 (0)
Metastatic	28 (100)
No. of disease sites (*n*, %)	
1	2 (7)
2	10 (36)
≥3	16 (57)
Mutational status (*n*, %)	
BRAF V600E	4 (14)
NRAS Q61R	3 (11)
NRAS Q61H	1 (4)
HRAS	1 (4)
TSC1	1 (4)
CDKN1A	1 (4)
CDKN2A	1 (4)
PTEN	1 (4)
AKT1	1 (4)
Prior treatment (*n*, %)	
Surgery	28 (100)
RAI therapy	25 (89)
Radiation therapy	16 (57)
Chemotherapy	0 (0)
Tyrosine kinase inhibitor	15* (54)

PTC papillary thyroid carcinoma, FTC follicular thyroid carcinoma FVPTC follicular variant PTC, PDPTC poorly differentiated PTC, ATC anaplastic thyroid carcinoma, * all 15 patients were previously treated with sorafenib

Efficacy

All 28 patients enrolled in this study were eligible for efficacy analysis. Data on efficacy are presented in Table 2 and Figure 1.

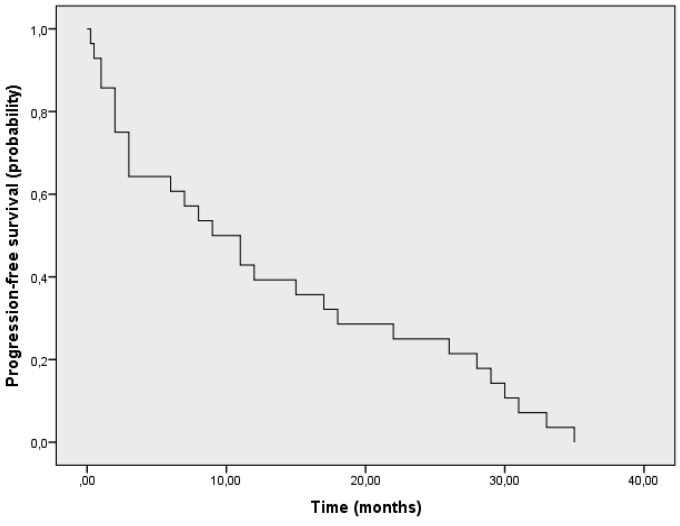
Seventeen patients (65%) showed stable disease (SD) as best response, with 15 (58%) having SD lasting >24 weeks. Median SD duration was 20 months (range 2-38 months). At the time of data analysis, 4 (15%) patients still had ongoing SD. Patients with high (>466 µg*hr/L) everolimus whole blood concentrations tended to have a longer median duration of SD (30 vs 11 months, p=0.053) than patients with low everolimus whole blood concentrations. There were no complete (CR) or partial (PR) responses. Estimated median progression free survival (PFS) was 9 months (95% CI: 4-14 months) and was longer in patients with high everolimus blood concentrations (22 vs 8 months, p=0.124). The median overall survival (OS) was 18 months (95% CI: 7-29 months, p=0.272) and was not significantly influenced by everolimus whole blood concentrations. However, the median OS was a substantial 15 months longer in patients with high everolimus whole blood concentrations. Furthermore, in patients with bone metastases, the median PFS was significantly worse with 10 vs. 30 months, p=0.001. Disease progression and survival were not influenced by age, gender or mutational status. Changes in thyroglobulin levels did not significantly reflect clinical outcome, however patients with PD showed a clear increase in serum Tg levels, as can be seen in figure 2.

Table 2 Efficacy analysis

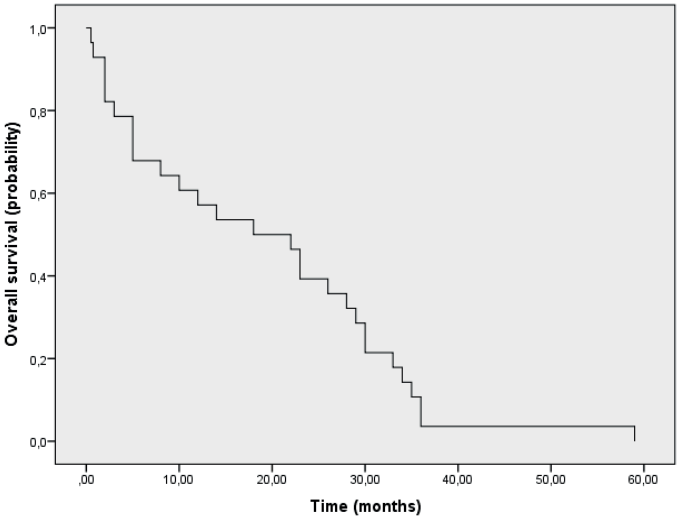
Parameter	*n=28*
Median duration of treatment (months; range)	11 (1-38)
Median duration of follow-up (months; range)	32 (2-64)
Best response by RECIST 1.0 (*n*, %)	
Complete response	0 (0)
Partial response	0 (0)
Stable disease	17 (65)†
Progressive disease	11 (39)
Overall disease control	17 (65)
Median duration of SD (months; range)	78 (9-150)‡
High everolimus blood concentration (months)	120
Low everolimus blood concentration (months)	45
Median PFS (months; 95% CI)	9 (3,8-14,2)
High everolimus blood concentration (months)	22
Low everolimus blood concentration (months)	8
Median OS (months; 95% CI)	18 (6,6-29,4)
High everolimus blood concentration (months)	29
Low everolimus blood concentration (months)	14
With bone metastases (months)	10
Without bone metastases (months)	30

RECIST Response Evaluation Criteria in Solid Tumors, SD stable disease, PFS progression-free survival, OS overall survival.
† 15 of 17 patients showed SD >24 weeks, ‡ at time of data analysis, 4 patients still had ongoing SD.

Figure 1 Kaplan-Meier curves of (A) progression-free survival and (B) overall survival and waterfall plot (C)



A



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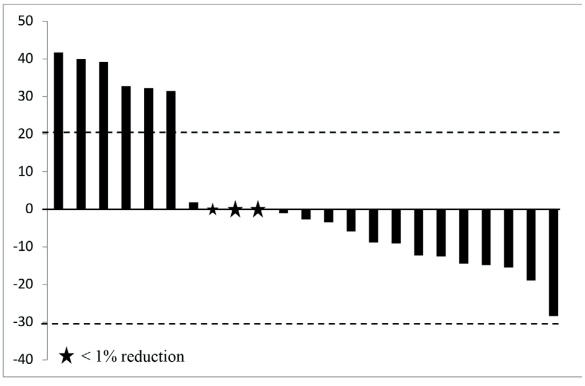
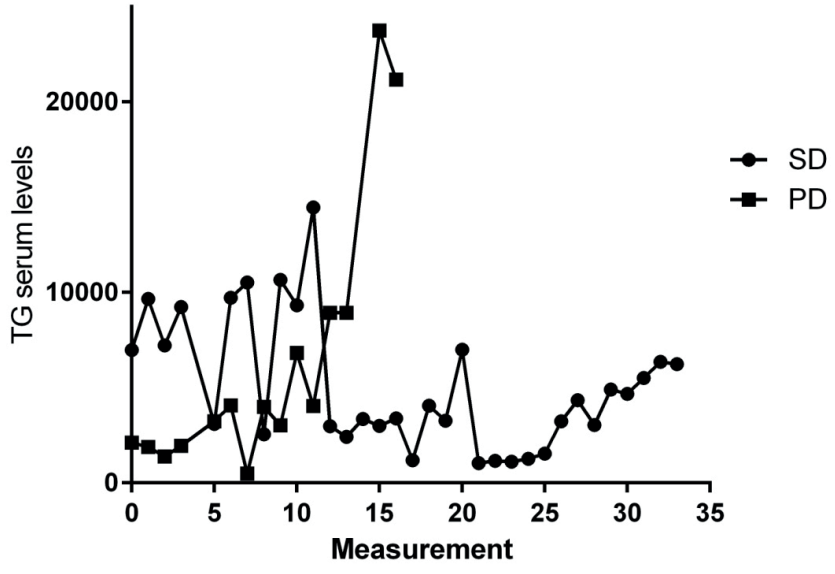


C Maximum reduction from baseline in the sum of the longest diameters of target lesions

Figure 2 Changes in thyroglobulin levels per cycle per patient response (SD vs. PD)



Mean thyroglobulin levels over time per patient response. SD stable disease, PD progressive disease

Toxicity

Thirteen patients (46%) required dose reduction due to toxicity (supplementary table 2). One patient (4%) discontinued everolimus treatment due to adverse events (AEs) as he developed kidney function disorders and anemia after 11 weeks of therapy. All observed AEs are listed in table 3. Treatment related AEs were predominantly grade 1 or 2, with the most common including anemia, cough, stomatitis and hyperglycemia. Grade 3 AEs consisted of stomatitis (11%), hypertension (11%), hypocalcaemia (7%), anemia (4%), hypophosphatemia (4%), anorexia (4%), and atelectasis (4%). Two grade 4 AEs were documented, 1 patient was hospitalized because of severe diarrhea and the other due to a pneumonia. A total of 10 serious AEs (SAEs) were reported, none of which could be related to everolimus treatment. The fast majority of AEs were controllable with dose reduction, medication or supporting measures. There was no relation between toxicity and performance state or age.

Table 3 Adverse events

	All	Grades Number of patients (% of category)			
Event	**Number of patients (% of total (*n*=28))**	**1**	**2**	**3**	**4**
Anemia	18 (64)	5 (28)	12 (67)	1 (5)	
Cough	18 (64)	17 (94)	1 (6)		
Stomatitis	17 (61)	12 (71)	2 (12)	3 (18)	
Hyperglycemia	17 (61)	7 (41)	7 (41)		
Hypertension	15 (54)	4 (27)	8 (53)	3 (20)	
Fatigue	14 (50)	11 (79)	3 (21)		
Hypophosphatemia	12 (43)	4 (33)	7 (58)	1 (8)	
Loss of apatite	11 (39)	8 (73)	3 (27)		
Thrombopenia	11 (39)	10 (91)	1 (9)		
Dyspnea	11 (36)	8 (73)	2 (18)	1 (9)	
Rash	10 (36)	8 (80)	2 (20)		
Peripheral edema	8 (29)	7 (88)	1 (12)		
Anorexia	8 (29)	3 (38)	4 (50)	1 (12)	
Pruritis	8 (29)	8 (100)			
Hypocalciemia	8 (29)	2 (25)	4 (50)	2 (25)	
Kidney function #	8 (29)	5 (63)	3 (37)		
Diarrhea	8 (29)	5 (63)	2 (25)		1 (12)
Nausea	7 (25)	7 (100)			
Other pulmonary	7 (25)	6 (86)		1 (14)	
Dry skin	6 (21)	6 (100)			
Haemorrhage	6 (21)	6 (100)			
Hypertryglyceridemia	6 (21)	3 (50)	3 (50)		
Infection	5 (18)	2 (40)	3 (60)		
Constipation	5 (18)	4 (80)	1 (20)		
Headache	5 (18)	5 (100)			
Dysphagia	4 (14)	3 (75)	1 (25)		
Hypercholesterolemia	4 (14)	1 (25)	1 (25)	2(50)	
Other cardiac	3 (11)	2 (66)	1 (33)		
Pneumonitis	3 (11)		2 (66)	1 (33)	
Liver function #	2 (7)		1 (50)	1 (50)	
Vomiting	2 (7)	2 (100)			
Fever	2 (7)	2 (100)			
Allergic reaction	2 (7)	1 (50)	1 (50)		
DVT	1 (4)	1 (100)			
Neutropenia	1 (4)		1 (100)		
Pneumonia	1 (4)				1 (100)

All AEs graded according to Common Terminology Criteria for Adverse Events version 4.

Somatic gene variant analysis

DNA for gene variant analysis was available for all 28 tumors. A total of 14 variants were identified in primary tumor material of 12 patients. Variants in *BRAF* were most frequently observed with 4/6 PTC patients harboring class 5 pathogenic *BRAF* variants (2 c.1799T>A, p.(Val600Glu), 1 c.1796T>A, p(Val600Glu) and one c.1799-1801het_delTGA). One of these patients also showed a class 4 *AKT1* c.49G>A, p.(Glu17Lys) pathogenic variant. A total of 4 class 5 pathogenic *NRAS* variants (one c.182A>G, p.(Gln61Arg), one c.183A>T, p.(Glu61His), one c.182A>G, p.(Gln61Arg) and one c.182A>G, p.(Glu61Arg)), one class 5 *CDKN1A*c.45_57del, p.15-19del pathogenic variant, one *CDKN2A* class 4 c.G250T, p.(Asp84Tyr) pathogenic variant and one class 5 heterozygous *TSC1* c.C163T, p.(Gln55X) pathogenic variant were seen. One patient harbored a class 5 *HRAS* c.182A>G, p.(Gln61Arg) pathogenic variant as well as a class 4 *PTEN* c.404T>A, p.(Ile135Lys) pathogenic variant. No significant relation between mutational status and response on everolimus treatment could be determined. The one patient with the *TSC1* variant started only seven weeks prior to his death, therefore the expected effects upon giving everolimus could not be monitored. However, 3/4 patients with a *NRAS* mutation and all 4 patients with a *BRAF* mutated tumor showed a PFS and OS longer than the mPFS and mOS respectively. For an overview of histology, mutational status and response to everolimus treatment see supplementary table 3.

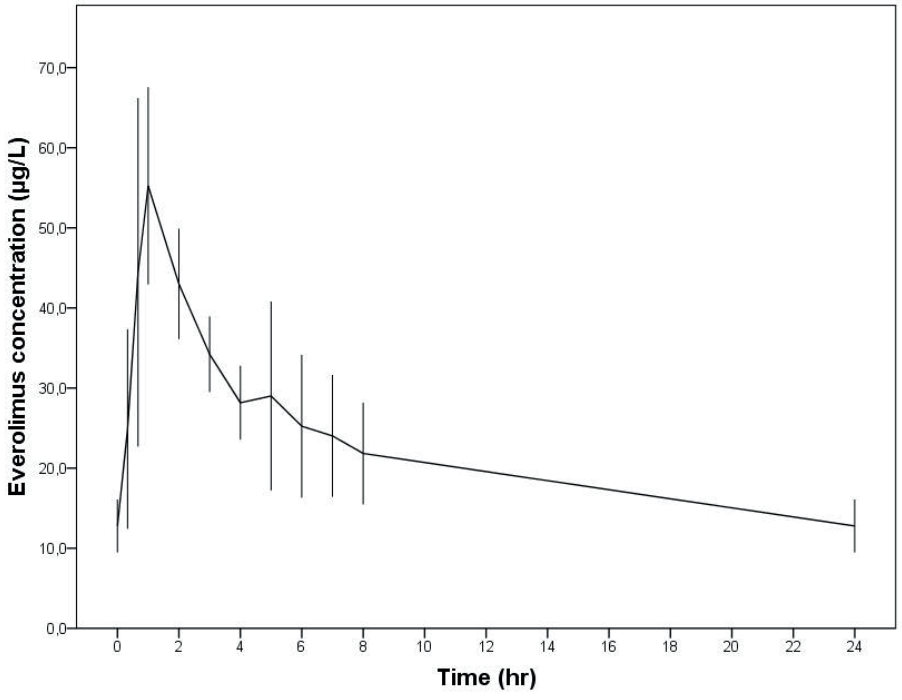
Pharmacokinetics

Four patients underwent the short PK sampling schedule; 22 patients participated in the extended sampling schedule. The mean concentration versus time profile with the 95%-CI intervals per time point is shown in Figure 3.

A summary of the everolimus pharmacokinetics is shown in supplementary table 4. The median (range) $AUC_{0\text{-}24hr}$ for everolimus was 466 µg·hr/L (259 - 1336 µg·hr/L) and the median C_{trough} was 9.8 µg/L (5.1 - 26.3 µg/L). The median T_{max} was 1.0 hours and the median C_{max} 63.6 µg/L. An independent samples Student's t-test showed no significant difference in $AUC_{0\text{-}24hr,}$ C_{trough}, T_{max} or C_{max} based on either the limited or extended sample scheduling, and we therefore combined the data from both groups as described above. Due to the relative scarcity of drug concentrations in the elimination phase, we only used data from patients with the extended curve (n=22) to calculate $T_{1/2}$. The median $T_{1/2}$ of everolimus in these patients was 18.9 hr (13.1 - 35.8 hr).

The relation between everolimus and toxicity in patients with thyroid cancer was recently reported in an everolimus PK analysis in a total of 42 patients with thyroid cancer of all histological subtypes. (26)

Figure 3 Mean everolimus whole blood concentration time



Bars represent the 95%-CI of the mean per time point.

DISCUSSION

This phase II study was designed to assess the efficacy, safety and PK of everolimus in patients with progressive radioiodine refractory unresectable recurrent or metastatic differentiated thyroid carcinoma. To our knowledge, this study has the longest follow-up duration compared to other studies of everolimus in patients with thyroid cancer (20,21,22,23). The results of this study are promising, with 58% of patients showing stable disease >24 weeks. The lack of PRs is easily explained by the fact that these tumors are slowly progressive by nature and everolimus exerts a reduced growth and proliferation effect rather than a cell death effect. The mPFS and OS were 9 and 18 months respectively. Interestingly, patients with high everolimus whole blood concentrations showed a longer median PFS, duration of SD and OS compared to patients with low concentrations, albeit not significant. Furthermore, we found a significantly worse OS in patients with bone metastases. Similar results have been reported in a phase II trial with sorafenib in patients with advanced DTC (27). The mPFS and percentage of patients with durable SD found in our study are comparable to the results of the phase

III DECISION trial of sorafenib in patients with advanced DTC (28). The phase III SELECT study of lenvatinib in patients with advanced DTC showed a mPFS of 73 weeks. The median OS was not reached, however our median follow-up duration was twice as long (29).

The results of the separately analyzed ATC patients were disappointing, with none of the 7 included patients showing response on everolimus treatment. Toxicity was comparable to other studies with everolimus in patients with thyroid cancer.

Somatic mutation status of genes in the mTOR pathway did not seem to stratify for response, even taken a patient with a *TSC1* somatic variant into account. Tumours that are dependent on the mTOR pathway might have increased sensitivity to mTOR inhibition and inactivating mutations in the tumour-suppressor genes *TSC1* and *TSC2* have been reported to result in mTOR-pathway activation (30-33). Recently a case report was published describing a patient with an advanced ATC harbouring a homozygous *TSC2* mutation with a near complete response on everolimus treatment that lasted for 18 months (22). Furthermore, a phase II study of everolimus in urothelial carcinoma reported a durable complete remission in a patient with a *TSC1* mutation (34). The fact that the patient with a *TSC1* mutation in our study did not show a good response on everolimus treatment is probably related to extend of disease and high progression rate at trial inclusion. In addition this could well be explained by the heterozygosity of the *TSC1* mutation. Although not significant, 7/8 patients with a *BRAF* or *NRAS* mutations showed a PFS and OS longer than the mPFS and mOS respectively. These findings correspond with previous studies, reporting on enhanced sensitivity to mTOR inhibition in a variety of *RAS/BRAF* mutated tumours (13,35-36). The lack of a relation between genetic analyses and response had also been observed in the decision and select trials. One of the explanations can be that genetic analyses have been performed in primary tumors that have been removed years before entering this study. The heterogeneity of genetic alterations in metastases in advanced DTC has been described recently (37).

Toxicities observed in our study were mostly grade 1 and 2 and similar to those reported in other phase II and III studies of everolimus(20-22,38-41). Adverse effects were in the majority of cases manageable with dose reduction, medication or supplementary measures. None of the SAEs were related to everolimus treatment.

The PK characteristics in our study are in line with those observed in other phase I pharmacokinetic studies investigating everolimus 10 mg once daily (42-45). The inter-patient variability in PK was also comparable to previous research.

Although everolimus is a promising agent in patients with a variety of solid tumors, it can be hypothesized that its inability to inhibit mTORC2 results in a less potent antitumor effect. An in vitro study showed simultaneous inhibition of mTORC2 during everolimus treatment resulted in greater efficacy and the prevention of requiring resistance (46). In addition, several studies describe a feedback loop between mTOR pathway and *RAS/MAPK/ERK* signaling, resulting in a different prosurvival signaling

pathway upon mTOR inhibition. Everolimus as a single target agent might therefore not be the most effective therapy, emphasizing the interest of studies with everolimus combination therapy (15,29,31,43-45).

In conclusion, given the disease control rate comparable to sorafenib, but everolimus' relatively low toxicity profile, we consider everolimus to be a promising agent in the combination treatment of patients with advanced differentiated thyroid cancer and further investigation of everolimus in sequential or combination therapy is warranted. Currently, trials combining everolimus with TKIs are being conducted (www.clinicaltrials.gov). With the exception of a spectacular result reported recently in the literature, in the majority of ATCs there seems to be no role for everolimus treatment.

Supplementary Table 1 Results of everolimus in patients with ATC

Study no	Gender	Age (years)	Treatment duration (wks)	Best response	Time till PD (wks)	Time till death (wks)	Toxicity
36	Female	68	12	PD	12	12	Diarrhea gr1 Anemia gr1 Hypertriglyceridemia gr 3 Hyperglycemia gr 2 Cough gr1
37	Male	76	9	PD	9	11	Hypertension gr2 Fatigue gr1
38	Female	48	4	PD	4	5	Fatigue gr2
39	Female	80	10	PD	9	14	Anemia gr1 Hyperglycemia gr1
40	Female	71	13	PD	12	24	Stomatitis gr1 Anorexia gr2 Fatigue gr1 Anemia gr2 Hyperglycemia gr1 Cough gr1
41	Male	54	8	PD	8	12	Skin rash gr1 Hypophosphatemia gr1
42	Female	49	13	PD	13	53	Stomatitis gr1 Anorexia gr1 Fatigue gr1 Hypertriglyceridemia gr 2 Hypocalcaemia gr1 Liverfunction # gr1 Cough gr1

Wks weeks, PD progressive disease, gr grade

Supplementary Table 2 Reason for dose reduction and time point of onset

Patient study no.	Toxicity	No. of treatment cycles before onset
1	Pneumonitis gr.2	10
2	Stomatitis gr.3	1
4	Stomatitis gr.2	1
5	Fatigue gr.2	15
7	Pneumonitis gr.2	6
11	Pneumonitis gr.3	2
13	Stomatitis gr.2	1
15	Anorexia gr.2	3
17	Stomatitis gr.3	1
18	Fatigue gr.2	1
21	Fatigue gr.2	3
22	Kidney function disorder gr.2	1
26	Liver function disorder gr.3	1

Supplementary Table 3 Mutational related outcomes

Study no.	Histology	Mutation	PFS (wks)	OS (wks)
1	PTC	*BRAF* c.1799-1801het_delTGA, p(Val600Glu)	78	95
4	PTC	*BRAF* c.1796T>A, p(Val600Glu)	97	158
8	FTC	*NRAS* c.182A>G, p.(Gln61Arg)	28	102
9	FTC-OV	*CDKN2A* c.G250T, p.(Asp84Tyr)	11	20
16	PTC	*BRAF* c.1799T>A, p(Val600Glu)	143	143*+
18	PTC	*TSC1* c.C163T, p.(Gln55X)	6	7
19	FTC	*CDKN1A* c.45_57del, p.15-19del	1	1
21	FVPTC	*NRAS* c.183A>T, p.(Glu61His)	128	128*+
22	FTC	*NRAS* c.182A>G, p.(Gln61Arg)	127	127*+
23	FVPTC	*NRAS* c.182A>G, p.(Glu61Arg)	67	122*
24	FTC	*HRAS* c.182A>G, p.(Gln61Arg) *PTEN* c.404T>A, p.(Ile135Lys)	41	113*
25	PTC	*BRAF* c.1799T>A, p.(Val600Glu) *AKT1* c.49G>A, p.(Glu17Lys)	113	113*+

PFS progression free survival, wks weeks, OS overall survival, PTC papillary thyroid carcinoma, FTC follicular thyroid carcinoma, FTC-OV follicular thyroid carcinoma oncocytic type, FVPTC follicular variant papillary thyroid carcinoma, * patient still alive at time of data analysis, + patient still on everolimus treatment at time of data analysis

Supplementary Table 4 Summary of everolimus pharmacokinetic parameters

	Median	Range	CV%
$AUC_{0\text{-}24hr}$ (µg·hr/L)	466	259 - 1336	48.0%
C_{trough} (µg/L)	9.8	5.1 - 26.3	56.9%
T_{max} (hr)	1.0	0.7 - 5.0	75.1%
C_{max} (µg/L)	63.6	34.8 - 116.9	39.9%
$T_{1/2}$ (hours)	18.9	13.1 - 35.8	32.6%

AUC, area under the concentration time curve; C_{trough}, minimum plasma concentration; C_{max}, peak plasma concentration; CV%, coefficient of variation, $T_{1/2}$, elimination half-life; T_{max}, time to reach peak plasma concentration.

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7

GENERAL DISCUSSION

DISCUSSION

In this thesis new treatment modalities are described in differentiated (DTC), anaplastic (ATC) and advanced medullary thyroid cancer (MTC). A large majority of patients with DTC can be cured with standard treatments, and others may survive long after initial disease diagnosis and treatment despite persistence of disease. However, DTC patients with progressive, radioiodine-resistant metastatic disease have a worse prognosis and the results of conventional treatment modalities (radiotherapy and/or chemotherapy) have been disappointing. These patients should nowadays be considered for entry into clinical trials with new biological agents. With new agents currently available, also patients with advanced MTC seem to have a much better perspective. For ATC patients, hopefully novel therapies will soon come available, as until now this devastating disease has shown to be almost resistant to any therapy. Advances in the treatment of persistent thyroid cancer is merely based on the molecular unraveling of the carcinogenesis pathways implicated in thyroid cancer. Aberrations in already classic cellular signal transduction pathways (e.g. RAS/RAF/MAPK) are present in high percentages of thyroid cancer.[1-3] Furthermore, angiogenesis pathways are mostly activated. Involvement of receptor tyrosine kinases, such as EGFR or c-Met were elucidated.[4] In addition, activating *RET* mutations are present in >95% of hereditary MTC and in 20–50% of sporadic MTC.[5] Because of the oncogenic roles of activated BRAF, RET, and RET/PTC kinases, the assumption was that specific targeting of these kinases could block tumor growth and induce senescence. As shown in multiple clinical trials targeting thyroid cancer, these ideas have to some extend appeared to be correct. Several promising agents were found for DTC and MTC.[6-43] However, the search for new agents that can be used in sequential or combination therapy will still be necessary, since many patients eventually become progressive using the now available biologicals or do not tolerate them. The recently much debated immunotherapy using immune checkpoint de-blocking might be of value in a small subset of patients with high PD-L1 expression in their thyroid cancer, although the mutational burden is relatively low in the majority of thyroid cancers. High mutational burden has shown to be correlated with efficacy of immune de-blocking agents in many human tumor types.[44-46] Currently a phase 1b clinical trial with pembrolizumab, an anti-PD-1 antibody, is being conducted and preliminary results are promising (NCT02054806).[47]

However, there are some issues that need attention. A substantial proportion of patients (33%-50%) have stable disease of varying duration as best response in several studies. Given the indolent natural history of many of these tumors, a report of stable disease is of limited value. Objective response rates give information on progression, stabilization or shrinkage of tumors within a certain period of time, but do not correlate per se with overall survival. Progression-free survival or overall survival are not always

preferred primary endpoints in advanced thyroid cancer because of its indolent nature. Furthermore, the RECIST-criteria may not be the best method for tumor evaluation in this tumor type since anatomical imaging alone may have limitations. Only patients with documented progressive disease should be included in clinical trials. If this is not the case, the value of achieving stable disease is even further undermined and thereby differences in outcomes between studies may develop. For determining any lack of progression by changes in tumor size PET/CT evaluation may be of more value. Despite the rapid integration of PET with 18F-FDG in the clinical practice and the development of the PET Response Criteria in Solid Tumors (PERCIST 1.0), there has been relatively little systematic integration of PET into clinical trials of new cancer treatments.[48] The role of FDG-PET/CT in the evaluation of thyroid cancer patients remains unclear. Carr et al. reported a decrease in FDG uptake to be associated with subsequent tumor response and an increase with tumor progression in DTC patients treated with sunitinib.[49] However, similar results in patients treated with vandetanib could not be determined. [50] Furthermore, it can be suggested that the ^{124}I PET/CT may be more sensitive in case of thyroid cancer. A recent study reported a sensitivity of 92.5% and a 22.5% increased detection of RAI-avid lesions by 124I PET/CT compared with the planar ^{131}I post-treatment scans.[51] Therefore, more research is needed to investigate the value of PET/CT in the treatment of advanced thyroid cancer, preferably in clinical trials.

Nowadays, mutational DNA and gene fusion screening can be performed on a routine basis in patients with (thyroid) cancer. Treatment with specific inhibitors could be initiated once actionable gene variants are present. So far, in relatively large studies of patients with advanced thyroid cancer, like the SELECT and DECISION trials, have not shown *RAS* or *BRAF* mutation related outcomes.[22,36,37] However, further research is needed for defining the clinical utility of molecular profile adjusted therapies in thyroid cancer and also in other sorts of cancer. In cooperation with The Dutch Center for Personalized Cancer Treatment (CPCT), the Drug Rediscovery Protocol (DRUP) study to determine the potential efficacy of targeted anti-cancer drugs in the treatment of patients with advanced cancers and a known molecular profile, is being performed (NCT02925234). In these studies, patients with advanced cancers who have exhausted standard treatment options, will be treated with commercially available targeted therapy matched to the patient's molecular tumor profile.

The developments in the treatment of advanced thyroid cancer are intriguing. The unraveling of the molecular pathways in thyroid cancer has played a pivotal role in the development of targeted therapy for thyroid cancer. Since the knowledge of the biological basis of thyroid cancer has increased, systemic treatment options for metastatic DTC and MTC have changed. A great variety of treatment strategies exists for patients with extensively metastasized thyroid cancer, although this type of cancer is rare and often has a slowly progressive course. The evidence for these different treatment strategies is limited. To date, sorafenib and lenvatinib are available as

standard systemic treatment for patients with
progressive, RAI refractory DTC and vandetanib and cabozantinib for patients with advanced MTC. However, in case of slowly progressive disease, the side effects of systemic treatment can outweigh the potential benefits. Given the low incidence of (metastatic) thyroid carcinoma, its slowly progressive course and the potential toxicity of targeted therapies, the timing of initiation of therapy is a delicate issue. It is of great importance to take the natural course of disease into account and to value not only possible benefits but also toxicity before starting targeted therapy. Hence, initiation of systemic therapy preferably has to be discussed with a specialized centre.

In conclusion, issues that need to be further addressed in advanced thyroid cancer are several fold: Which radiological examination should be best used for detection and monitoring of advanced disease? Which response evaluation criteria are most suitable? What is the optimal timing of starting therapy and which primary endpoint should be used in clinical studies? Furthermore, to increase the accrual of advanced thyroid cancer patients in clinical studies, to optimize the design of protocols, to improve the characterization of tumor tissues, and to improve the tolerance of treatment, a multidisciplinary team of medical oncologists, endocrinologists, specialists in nuclear medicine, radiologists, surgeons, pathologists, molecular biologists and statisticians is highly recommended.

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8

SUMMARY

SUMMARY

The general introduction in **Chapter 1** presents clinical aspects and molecular backgrounds of advanced thyroid cancer. In this review we have described new treatment modalities in advanced non-medullary differentiated thyroid cancer (DTC), anaplastic thyroid cancer (ATC) and advanced medullary thyroid cancer (MTC). A large proportion of patients with DTC are generally completely cured after surgery and subsequent radioactive iodine (RAI) therapy. Others may survive for decades despite persistent disease. However, DTC patients with progressive RAI-resistant metastatic disease have a worse prognosis and the results of conventional treatment modalities (radiotherapy and/or chemotherapy) have been disappointing. These patients should nowadays be considered for entry into clinical trials with new agents. With the new agents currently available, also advanced MTC patients seem to have better perspectives. For ATC patients, hopefully, combinations of targeting biologicals and/or immunotherapy treatment modalities might pose better treatment options since this devastating disease has shown to be almost therapy resistant.

In **Chapter 1** signal transduction and angiogenesis pathways are described that are operational in thyroid cancer and which form the "drivers" of such lesions. As for other human cancers a wave of optimism filled the oncology field with ideas that specific blockade of such pathways would be the way to stop tumor growth, induce apoptosis or senescence and obtain cure. These assumptions have partially appeared to be correct. Several promising agents have been identified to be used in the treatment of non-medullary DTC and MTC. However, the search for optimal protocols to be used in sequential or in combination therapy is necessary since patients eventually show progressive disease upon using such targeting drugs or do not tolerate them. Not part of this thesis is the important question whether the loss of iodine transport can be reinstalled upon giving targeting treatments. This thesis is divided in two parts: a section dealing with the multi-kinase inhibitor sorafenib and a part with the mTOR pathways inhibitor everolimus.

PART 1: SORAFENIB

Sorafenib (BAY-43-9006) is an orally active multikinase tyrosine kinase inhibitor (TKI) that activates antiangiogenic and pro-apoptotic pathways, targeting the Btype Raf kinase (BRAF), vascular endothelial growth factor receptors (VEGFR) 1 and 2, and rearranged during transfection (RET). Sorafenib is widely approved for the treatment of patients with hepatocellular carcinoma (HCC) and advanced renal cell carcinoma (RCC) in well-defined phases of disease. **Chapter 2** describes the long-term results of a prospective phase II clinical trial to determine the efficacy of sorafenib in patients with advanced RAI refractory non-medullary DTC and MTC. Thirty-one patients with progressive metastatic or locally advanced radioactive iodine refractory differentiated thyroid cancer received sorafenib 400 mg orally twice daily. The study end points included response rate, progression-free survival (PFS), overall survival (OS), best response by Response Evaluation Criteria in Solid Tumors criteria 1.0, and toxicity. The Median PFS was 18 months (95% confidence interval (95% CI): 7-29 months) and median OS was 34.5 months (95% CI: 19-50 months). Eight patients (31%) achieved a partial response and 11 patients (42%) showed stable disease after a median follow-up of 25 months (range 3.5-39 months). Data of multiple clinical trials showed that sorafenib has a generally well-tolerated profile of adverse events (AEs). The most commonly reported sorafenib related AEs in DTC include hand-foot syndrome, hypertension, weight loss, diarrhea and rash. Recent reports have also suggested a possible causal link between Sorafenib therapy and the development of cutaneous squamous cell carcinomas (SCC). In conclusion, this study showed sorafenib to have clinically relevant antitumor activity in patients with progressive metastatic or locally advanced non-medullary RAI refractory DTC. Sorafenib can nowadays be considered as a standard option in these patients and since June 2015 sorafenib is also registered for the treatment of patients with thyroid cancer by the
European Medicines Agency (EMA).

In **Chapter 3** we describe three patients with a history of sorafenib treatment for advanced radioactive iodine refractory papillary thyroid cancer (two with a *BRAF* c.1799 T>A and one carrying a rare c.1799-1801het_delTGA mutation) who presented with secondary non-cutaneous lesions. The first patient was diagnosed with a squamous cell carcinoma (SCC) of the tongue, the second patient with a primary adenocarcinoma of the lung, and the third with a SCC originating from the cricoid. Secondary analysis was required to show that the latter two presentations were in fact recurrent thyroid cancers. These findings suggest that drugs such as sorafenib may induce metaplasia/clonal divergence of metastatic thyroid cancer and thus cause diagnostic misclassification. Furthermore, sorafenib is potentially involved in the tumorigenesis of secondary non-cutaneous SCC. These observations should now be confirmed in larger series of patients treated with drugs such as sorafenib.

PART 2: EVEROLIMUS

Until recently, surgery was accompanied by only limited curative and palliative treatment options for patients with MTC, emphasizing the need for new therapies. The mTOR inhibitor everolimus has shown encouraging results in the treatment of neuroendocrine tumors. Using everolimus, a significant dose-dependent inhibition in cell proliferation was observed in two medullary thyroid cancer cell lines [11]. As part of a prospective phase II study, we report on the safety and efficacy of everolimus in advanced MTC in **Chapter 4**. Seven patients with per RECIST 1.1 documented advanced MTC were included and received everolimus 10mg daily. The primary objective was determining treatment efficacy. Secondary endpoints included progression-free survival (PFS), overall survival (OS), toxicity, and pharmacokinetics (PK). The median follow-up duration was 28 weeks (17–147). Five patients (71%) showed SD, of which 4 (57%) showed SD>24 weeks. Median PFS and OS were 33 (95%CI: 8–56) and 30 (95%CI: 15–45) weeks, respectively. Toxicity was predominantly grade 1/2 and included mucositis (43%), fatigue (43%), and hypertriglyceridemia (43%). Four MTCs harbored the somatic *RET* mutation c.2753T>C, p.Met918Thr.The best clinical response was seen in a MTC patients with Multiple Endocrine Neoplasia type 2A patient. PK characteristics were consistent with phase I data. One patient exhibited extensive toxicity accompanying elevated everolimus plasma concentrations. In conclusion, this study suggests that everolimus exerts clinically relevant antitumor activity in patients with advanced MTC. Given the high rate of clinical benefit and the relatively low toxicity profile found in this MTC subgroup analysis, we believe that further investigation in larger cohorts of MTC patients is now warranted, using everolimus either as a single agent or in sequential or combination therapy.

Many patients treated with the registered fixed 10 mg dose of everolimus once daily are in need of dose interruptions, reductions or treatment discontinuation due to severe adverse events. In **Chapter 5** we determined the correlation between systemic everolimus exposure and toxicity. Additionally, the effect of different covariates on everolimus pharmacokinetics (PK) was explored. Forty-two patients with advanced thyroid carcinoma were treated with 10 mg everolimus once daily. Serial pharmacokinetic sampling was performed on days 1 and 15. Subsequently, a population PK model was developed using NONMEM to estimate individual PK values used for analysis of an exposure–toxicity relationship. Furthermore, this model was used to investigate the influence of patient characteristics and genetic polymorphisms in genes coding for enzymes relevant in everolimus PK. Patients who required a dose reduction (n =18) due to toxicity at any time during treatment had significant higher everolimus exposures [mean AUC0–24 (SD) 600 (274) vs. 395 (129) µg h/L, P = 0.008] than patients without a

dose reduction (n = 22). A significant association between Everolimus exposure and stomatitis was found (P = 0.047). The presence of at least one TTT haplotype in the *ABCB1*gene was associated with a 21 % decrease in everolimus exposure. In conclusion, this current study showed that dose reductions and everolimus-induced stomatitis were strongly associated with systemic everolimus drug exposure in patients with thyroid cancer. Our findings suggest that everolimus is a good candidate for individualized dosing in patients.

Chapter 6 presents the results of a prospective phase II clinical trial to determine the efficacy and safety of everolimus in patients with advanced follicular-derived thyroid cancer. Twenty-eight patients with progressive metastatic or locally advanced radioactive refractory follicular-derived thyroid cancer and 7 patients with anaplastic thyroid cancer were included and received everolimus 10mg orally once daily. The primary endpoint was disease control rate (complete (CR) + partial response (PR) + stable disease (SD) >24 weeks). Secondary endpoints included progression free survival (PFS), overall survival (OS), toxicity, mutational related outcomes and pharmacokinetic related outcomes (PK). Results of this study in patients with ATC were disappointing, with none of the patients benefiting from treatment. In the follicular-derive group, median follow-up duration was 38 months (2-64). Seventeen patients (65%) showed SD, of which 15 (58%) showed SD > 24 weeks. No CR or PR were observed. Median PFS and OS were 9 (95%CI:4-14) and 18 (95%CI:7-29) months, respectively. Survival was negatively influenced by the presence of bone metastases. Toxicity was predominantly grade 1/2 and included anemia (64%), cough (64%), stomatitis (61%) and hyperglycemia (61%). Duration of SD was related to everolimus exposure. The presence of somatic gene variants related to mTOR signaling did not clearly stratify for responses. In conclusion, with the exception of a spectacular result reported recently in the literature, there seems to be no role for everolimus treatment in the majority of ATCs. However, given the disease control rate comparable to sorafenib, but everolimus' relatively low toxicity profile, we consider everolimus to be a promising agent in the combination treatment of patients with advanced differentiated thyroid cancer and further investigation of everolimus regimens in sequential or combination therapy is warranted.

9

NEDERLANDSE SAMENVATTING

SAMENVATTING

De algemene introductie in **Hoofdstuk 1** geeft een overzicht van de klinische en moleculaire aspecten van het gevorderde schildkliercarcinoom. In dit overzichtsartikel beschrijven we de nieuwe behandelingsmogelijkheden van gedifferentieerd non-medullair schildkliercarcinoom (NMTC), anaplastisch (ATC) en medullair schildkliercarcinoom (MTC). Het overgrote deel van de patiënten met gedifferentieerd NMTC kan worden genezen met de combinatie van operatie en radioactief jodium (RAI) nabehandeling, anderen leven nog tientallen jaren ondanks aanhoudende ziekte. Patiënten waarbij operatie niet meer zinvol is of de tumorcellen geen RAI meer opnemen hebben echter een slechtere prognose en de werkzaamheid van bestraling/ chemotherapie is teleurstellend. Het moet dan ook overwogen worden deze patiënten te includeren in klinische studies waarbij nieuwe geneesmiddelen worden getest. Met de inmiddels beschikbare nieuwe behandelingsmogelijkheden lijken ook patiënten met gevorderde MTC betere overlevingskansen te hebben. Helaas bestaan er tot op heden nog geen effectieve behandelopties voor het lokaal uitgebreide of gemetastaseerde ATC.

De grote ontwikkelingen die de afgelopen jaren hebben plaatsgevonden op het gebied van de behandeling van het gevorderde schildkliercarcinoom zijn te danken aan de toenemende kennis van de biologische basis die ten grondslag ligt aan het ontstaan van deze maligniteit. Afwijkingen in onder andere de zogenaamde RET/PTC-RAS-RAF-MAPK tumoroutes zijn aanwezig in een hoog percentage van de schildkliertumoren, waaronder veranderingen in “tyrosine-kinases (TKs)” betrokken bij tumorgroei en vaatgroei zoals EGFR en c-Met. Vanwege de oncogene rol van geactiveerde BRAF, RET en RET / PTC kinases bestond de veronderstelling dat gericht blokkeren van deze kinasen tumorgroei zou kunnen afremmen cq stoppen. Zoals blijkt uit meerdere klinische studies bij patiënten met schildklierkanker, zijn deze veronderstellingen deels correct. Verscheidene veelbelovende middelen zijn de afgelopen jaren ontwikkeld voor de behandeling van gevorderd DTC en
MTC. Toch zal de zoektocht naar nieuwe therapieën in opeenvolgende of combinatietherapie nog steeds nodig zijn, aangezien patiënten uiteindelijk toch opflakkerende ziekte tonen of zijn de bijwerkingen niet (meer) te verdragen. Mogelijk kunnen deze gerichte therapieën ook de gevoeligheid voor RAI behandeling herstellen. Dit werd niet onderzocht in dit proefschrift. Het werk beschreven in dit proefschrift valt uiteen in twee delen: Enerzijds een deel betreffende het gebruik van de remmer sorafenib, anderzijds het gebruik van het middel everolimus.

DEEL 1: SORAFENIB

Sorafenib is een TK-remmer die celdeling en vaatgroei remt en door in te grijpen op het molecuul "BRAF", "vascular endothelial growth factor receptor" (VEGFR) types 1 en 2 en "*rearranged during transfection"* (RET). Sorafenib was al geregistreerd voor de behandeling van patiënten met gevorderde leverceltumoren en nierceltumoren en heeft inmiddels ook een registratie voor het lokaal vergevorderde of gemetastaseerde DTC. **Hoofdstuk 2** beschrijft de lange termijn resultaten van een klinische studie naar de werkzaamheid van sorafenib in patiënten met een gevorderd NMTC, niet meer gevoelig voor RAI. Een-en-dertig patiënten met uitgezaaid of in de hals aanwezig tumorresten werden behandeld met 400 mg sorafenib tweemaal daags. De uitkomsten werden gescoord op verschillende klinische parameters, waaronder effect van behandeling, overleving en bijwerkingen. Acht patiënten (31%) lieten een gedeeltelijke reactie zien en 11 patiënten (42%) bereikten stabiele ziekte na een mediane follow-up duur van 25 maanden (3.5-39). Bijwerkingen bestonden voornamelijk uit zenuwpijnen in handen en voeten, gewichtsverlies, diarree en huiduitslag. Recente data suggereren echter ook een mogelijk verband tussen het gebruik van sorafenib en het ontstaan van bepaalde huidtumoren. Concluderend liet onze studie zien dat sorafenib relevante tumor remming geeft bij patiënten met een vergevorderd NMTC. Sorafenib kan tegenwoordig worden beschouwd als een standaard behandeloptie bij
deze patiënten en is sinds juni 2015 door de European Medicines Agency (EMA) geregistreerd voor de behandeling van patiënten met gevorderd, RAI-ongevoelig gedifferentieerd NMTC.

In **Hoofdstuk 3** beschrijven we drie patiënten met een voorgeschiedenis van sorafenib therapie vanwege een gevorderd RAI ongevoelig NMTC (twee hiervan toonden een *BRAF* c.1799 T>A genverandering en één een zeldzame c.1799-1801het_delTGA BRAF genverandering) waarbij zich andere tumoren openbaarden. De eerste patiënt werd gediagnosticeerd met een tumor van de tong, de tweede patiënt met een longtumor, en de derde met een strottenhoofdtumor. Herhaalde tumoranalyse in ons instituut gebruikmakend van de nieuwste technieken toonde echter dat de tumoren bij de laatste twee patiënten in feite teruggekeerde schildklierkanker betrof. Deze bevindingen lijken te suggereren dat bij behandeling met geneesmiddelen zoals sorafenib veranderingen in de tumoren optreden waardoor deze bij standaard analyse niet meer goed worden herkend. Patiënten lopen dan het risico verkeerd behandeld te worden. Deze waarnemingen zullen nauwlettend moeten worden geanalyseerd in grotere series van kankerpatiënten met een voorgeschiedenis van behandeling met geneesmiddelen zoals sorafenib.

DEEL 2: EVEROLIMUS

Tot voor kort ging de operatieve behandeling van gevorderd MTC gepaard met beperkteverlenging van de levensverwachting voor de betrokken patiënten. Het geneesmiddel "*mammalian target of rapamycin (mTOR) inhibitor*" everolimus toonde eerder bemoedigende resultaten in de behandeling van neuroendocriene tumoren. Bovendien werd een aanmerkelijke remming van celproliferatie waargenomen in twee MTC cellijnen. Als onderdeel van een prospectieve fase II studie rapporteren we de effectiviteit en veiligheid van everolimus in gevorderd MTC in **Hoofdstuk 4**. Zeven patiënten met een vergevorderd MTC werden geïncludeerd en eenmaal daags met 10 mg everolimus behandeld. De uitkomstmaten betroffen wederom verschillende klinische gegevens, als genoemd bij de sorafenib behandeling. Vijf patiënten (71%) lieten stabiele ziekte (SD) zien, waarvan 4 patiënten (57%) # 24 weken. Bijwerkingen betroffen voornamelijk slijmvliesontstekingen, moeheid en veranderingen in de stofwisseling. Vier MTCs toonde genveranderingen in het zogenaamde RET gen. De beste klinische respons werd gezien in een voor MTC erfelijke belaste patiënt lijdend aan het Multipele Endocriene Neoplasie syndroom type 2A. Eén patiënt toonde uitgebreide bijwerkingen naast abnormaal hoge bloedwaarden van everolimus bij deze patiënt. Concluderend suggereert deze studie dat everolimus klinisch relevante antitumor activiteit heeft in patiënten met gevorderd MTC. Gezien de hoge mate van klinisch voordeel en het relatief lage toxiciteitprofiel van everolimus is verder onderzoek in grotere patiëntengroepen naar de rol van everolimus, als monotherapie of in combinatietherapie, belangrijk voor de toekomst.

In **Hoofdstuk 5** is gekeken naar de correlatie tussen systemische blootstelling van everolimus en bijwerkingen in patiënten met schildklierkanker. Geneesmiddelen worden na opname omgezet, afgebroken en/of uitgescheiden. Dit kan verschillend zijn tussen patiënten en dit werd onderzocht. Tweeënveertig patiënten met een gevorderd schildkliercarcinoom werden behandeld met eenmaal daags 10 mg everolimus. Bloedmonsters werden afgenomen op dag 1 en 15. Vervolgens werd een model ontwikkeld waarbij bloedwaarden en bijwerkingen werden vergeleken. Ook werd er naar subtiele individuele genveranderingen gekeken in gene die betrokken zijn met de omzetting van geneesmiddelen in het lichaam. De resultaten lieten zien dat patiënten die vanwege bijwerkingen minder Everolimus moesten krijgen veel hogere bloedwaarden hadden dan patiënten waar dit niet het geval was. De bevindingen suggereren dat bij gebruik van het geneesmiddel everolimus individuele dosering op basis van bloedwaarden een reële optie is.

Hoofdstuk 6 presenteert de resultaten van een studie naar de effectiviteit en

veiligheid van everolimus in patiënten met DTC. Achtentwintig patiënten met uitgezaaid of in de hals resterende hoeveelheid NMTC ongevoelig voor RAI behandeling en 7 patiënten met ATC werden geïncludeerd en behandeld met 10 mg everolimus eenmaal daags. Er werd gekeken naar uitkomsten waaronder overleving, bijwerkingen en geneesmiddelomzettingen in het lichaam (zie boven). De resultaten van onze studie in de groep ATC patiënten waren teleurstellend daar geen enkele patiënt voordeel liet zien van behandeling met everolimus. Zeventien van de 28 patiënten (65%) in de NMTC groep toonden stabiele ziekte. Overleving bleek negatief beïnvloed te worden door de aanwezigheid van botmetastasen. Bijwerkingen betroffen bloedarmoede (64%), hoesten (64%), mondslijmvliesontsteking (61%) en hoge suikerspiegels in het bloed (61%). De duur van klinische effecten kon worden gerelateerd aan everolimus blootstelling. De aanwezigheid van allerlei ter zake doende genvarianten relateerde niet eenduidig voor de effecten. Concluderend, met uitzondering van een door anderen recent gerapporteerd spectaculair behandelingseffect bij een ATC patiënt, lijkt er geen rol te zijn voor everolimus in de behandeling van het ATC. Echter gezien de effectiviteit van everolimus die vergelijkbaar is met sorafenib, maar waarbij everolimus' relatief een geringe hoeveelheid bijwerkingen toont, beschouwen we everolimus als een veelbelovend middel in de combinatietherapie van patiënten met gevorderd gedifferentieerd NMTC. Verder onderzoek van everolimus in opeenvolgende of combinatietherapieën is in onze ogen zinvol en wenselijk.

CURRICULUM VITAE

Tatiana Schneider werd geboren op 3 september 1987 te Alkmaar. In 2005 behaalde zij haar Gymnasium diploma aan het Murmellius Gymnasium te Alkmaar. Daaropvolgend studeerde zij een jaar Biomedische Wetenschappen aan de Katholieke Universiteit Leuven in Belgie. In 2006 werd begonnen met de studie Geneeskunde aan de Universiteit Leiden. Tijdens haar studie was zij in de snijzaal actief als student-assistent bij de Vakgroep Anatomie. Haar keuze-coschap bracht zij door op de afdeling Kinderchirurgie van het Juliana Kinderziekenhuis en haar semi-arts stage op de afdeling Heelkunde van het Medisch Centrum Haaglanden. Het doctoraalexamen van de studie Geneeskunde werd behaald in 2010, het artsexamen in 2012. In Januari 2013 begon zij als arts-onderzoeker op de afdelingen Klinische Oncologie en Pathologie van het LUMC onder de leiding van Prof. van der Hoeven, Prof. Morreau en dr. Kapiteijn, hetgeen heeft geresulteerd in dit proefschrift. Van februari 2013 tot maart 2015 assisteerde zij op OK bij de Nederlandse Obesitas Kliniek, en van maart 2015 tot April 2016 was zij werkzaam als arts-assistent Heelkunde in het Medisch Centrum Haaglanden. In mei 2016 is zij gestart met de opleiding tot plastisch chirurg in het aan de Universiteit van Erlangen geaffilieerde Sana Klinikum Hof, Duitsland.

LIST OF PUBLICATIONS

2016: **Schneider TC,** Wit de D., Links T.P., Erp van N.P., Hoeven van der J.J.M, Gelderblom H., Roozen I, Bos M, Corver W.E., van Wezel T., Morreau H., Guchelaar H.J., Kapiteijn E. *Everolimus in patients with advanced follicular-derived thyroid cancer, results of a phase II clinical trial.* J Clin Endocrinol Metab 2017;102:698-707

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LIST OF ABBREVIATIONS

AE	adverse event
AKT	AKR mouse thymoma kinase
ALAT	alanine aminotransferase
ALK	anaplastic lymphoma kinase
ALT	alanine aminotransferase
ANC	absolute neutrophil count
ASAT	aspartat aminotransferase
ASCO	American Society of Clinical Oncology
AST	aspartate aminotransferase
ATC	anaplastic thyroid cancer
ATP	adenosine triphosphate
AUC	aria under the curve
BCR-ABL	breakpoint cluster region-Abelson
BM	bone metastases
BRAF	B-type Raf kinase
BSA	body surface area
CD	cluster of differentiation
CDKN	cyclin-dependent kinase inhibitor
CEA	carcinoembryonic antigen
CHEK2	checkpoint kinase 2
CI	confidence interval
CK	cytokeratin
c-KIT	mast/stem cell growth factor receptor
C_{max}	peak concentration
COV	coefficient of variation
CPCT	Center for Personalized Cancer Treatment
CR	complete response
CT	calcitonin
CT	computed tomography
CTCAE	common terminology criteria for adverse events
$C_{through}$	through drug concentration
CYP	cytochrome P
DNA	deoxyribonucleic acid
DRUP	drug rediscovery protocol
DTC	differentiated thyroid cancer
DVT	deep venous thrombose
EDTA	ethylene diamine tetra acetic acid
EGFR	endothelial growth factor receptor
ELM4	echinoderm microtubule-associated protein-like 4
EMA	European medicines agency

EPAR	European public Assessment Report
ERK	extracellular signal-regulated kinases
ESMO	European Society for Medical Oncology
F	bioavailability
FGFR	fibroblast growth factor receptor
FKBP	FK206 binding protein
FLCN	folliculin
FNA	fine needle aspirate
FOCE	first order conditional estimation
FTC	follicular thyroid cancer
FTC-OV	oncocytic thyroid carcinoma
FVPTC	follicular variant PTC
GFPT	glutamine-fructose-6-phosphate transaminase
HCC	hepatocellular carcinoma
HE	hematoxylin eosin
$HER2^-$	human epidermal growth factor-2 negative
HFS	hand foot syndrome
HIF	hypoxia inducible factor
HR	hazard ratio
HR^+	hormone receptor positive
HRAS	V-Ha-Ras Harvey rat sarcoma viral oncogene
hTERT	human telomerase reverse transcriptase
IC	inhibitory concentration
IHC	immunohistochemistry
KRAS	Kirsten rat sarcoma viral oncogene
LLOQ	lower limit of quantification
MAF	minor allele frequencies
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase
MET	hepatocyte growth factor receptor
MEN2A	multiple endocrine neoplasia type 2A
MEN2B	multiple endocrine neoplasia type 2B
mOS	median overall survival
mPFS	median progression free survival
mRCC	metastatic renal cell carcinoma
mRNA	messenger RNA
MTC	medullary thyroid cancer
mTOR	mammalian target of rapamycin
mTORC2	mammalian target of rapamycin complex 2
N	number
NCI	national cancer institute
NET	neuroendocrine tumor
NF	neurofibromatosis

NGF	nerve growth factor
NGS	next generation sequencing
NHG	near-homozygous genome
NMTC	nonmedullary thyroid cancer
NONMEM	nonlinear mixed-effects modeling
NR	not reached
NRAS	neuroblastoma rat sarcoma viral oncogene
NSCLC	non-small-cell lung cancer
NTRK	neurotrophic tyrosine kinase receptor
OFV	objective function value
OS	overall survival
PAX	paired box
PD	progressive disease
PD1	programmed cell death protein 1
PDGFR	platelet derived growth factor receptor
PD-L1	programmed death-ligand
1PDPTC	poorly differentiated papillary thyroid cancer
PDTC	poorly differentiated thyroid cancer
PET/CT	positron emission tomography and computed tomography
PFS	progression free survival
PGM	personal genome machine
P-gp	P-glycoprotein
PI3K	phosphatidylinositol-3-kinase
PK	pharmacokinetics
pNET	pancreatic neuroendocrine tumor
PPM1D	protein phosphatase 1D
PPARɣ	peroxisome proliferator-activated receptor gamma
PR	partial response
PRCSD	disease control rate
PTC	papillary thyroid cancer
PTEN	phosphatase and tensin homolog
Pts	patients
PXR	pregnane X receptor
qPCR	quantitative real-time PCR
RAF	rapidly accelerated fibrosarcoma
RAI	radioactive iodine
RAS	rat sarcoma
RCC	renal cell carcinoma
RECIST	Response Evaluation Criteria in Solid Tumors
RET	rearranged during transfection
ROS1	c-ros oncogene 1
RR	response rate
RSE	relative standard error

RTK	receptor tyrosine kinase
SAE	serious adverse event
SCC	squamous cell carcinoma
SD	stable disease
SD	standard deviation
SEGA	subependymal giant cell astrocytoma
SNP	single nucleotide polymorphism
SPSS	statistical package for the social sciences
STRN	striatin
$T_{1/2}$	elimination halftime
T4	free thyroxine
TC	thyroid cancer
TDM	therapeutic drug monitoring
TERT	telomerase reverse transcriptase
TFG	transforming growth factor
Tg	thyroglobulin
TK	tyrosine kinase
TKI	tyrosine kinase inhibitor
T_{max}	time to reach peak concentration
TNM	tumor node metastases
TP53	tumor protein 53
TRK	tropomyosin receptor kinase
TSC	tuberous sclerosis complex
TSH	thyroid stimulating hormone
TTF	thyroid transcription factor
ULN	upper limit of normal
UPLC-MS	ultra performance liquid chromatography-tandem mass spectrometry
VEGF	vascular endothelial growth factor
VEGFR	VEGF receptor
VPC	visual predictive check
WBC	white blood count

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