REVIEW

Tetrac as an anti-angiogenic agent in cancer

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Abstract

The thyroid hormones T3 and T4 have emerged as pro-angiogenic hormones with important implications for cancer management. Endogenous circulating hormone levels may help stimulate cancer progression and limit the effectiveness of anticancer therapy, though clinical data remain inconclusive. The capacity of thyroid hormones to modulate angiogenesis is mediated through non-canonical mechanisms initiated at the cell surface receptor integrin $\alpha\beta 3$. This integrin is predominantly expressed on tumour cells, proliferating endothelial cells and tumour stroma-associated cells, emphasising its potential relevance in angiogenesis and tumour biology. Thyroid hormone/integrin $\alpha\beta 3$ signalling results in the activation of intracellular pathways that are commonly associated with angiogenesis and are mediated through classical pro-angiogenic molecules such as vascular endothelial growth factor. The naturally occurring T4 analogue tetrac blocks the pro-angiogenic actions of thyroid hormones at the integrin receptor, in addition to agonist-independent anti-angiogenic effects. Tetrac reduces endothelial cell proliferation, migration and tube formation through a reduction in the transcription of vascular growth factors/growth factor receptors, hypoxia-inducible factor-1 α , pro-angiogenic cytokines and a number of other pro-angiogenic genes, while at the same time stimulating the expression of endogenous angiogenesis inhibitors. It further modulates vascular growth factor activity by disrupting the crosstalk between integrin $\alpha\beta 3$ and adjacent growth factor receptors. Moreover, tetrac disrupts thyroid hormone-stimulated tumour recruitment, differentiation and the pro-angiogenic signalling of tumour stroma-associated mesenchymal stem cells. Tetrac affects tumour-associated angiogenesis via multiple mechanisms and interferes with other cancer cell survival pathways. In conjunction with its low toxicity and high tissue selectivity, tetrac is a promising candidate for clinical application.

Key Words

- ▶ tetrac
- ▶ integrin $\alpha\beta 3$
- ▶ thyroid hormones
- ▶ angiogenesis
- ▶ cancer

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Introduction

The thyroid hormones 3,5,3'-triiodo-L-thyronine (T3) and L-thyroxine (T4) are essential regulators of development, differentiation, growth and metabolism, both in the developing and the adult organism (Brent 2012, Davis *et al.* 2018). Given that they influence such a broad range of processes, it is not surprising that growing clinical and experimental evidence suggests that they are also implicated in cancer development and progression,

affecting cancer cell proliferation, cancer cell defence pathways and angiogenesis (Davis *et al.* 2014b). These effects are thought to be mediated in large part by a non-canonical pathway initiated at the extracellular domain of the plasma membrane protein integrin $\alpha\beta 3$, first described by Paul Davis and co-workers (Bergh *et al.* 2005). Integrin $\alpha\beta 3$ is primarily expressed on cancer cells, dividing endothelial cells and tumour

stroma-associated cells, thus highlighting its potential importance in oncology (Dijkgraaf *et al.* 2009, Schmohl *et al.* 2015). 3,5,3',5'-tetraiodothyroacetic acid (tetrac), a naturally occurring metabolite of T4, specifically blocks the binding and thyroid hormone action at the integrin receptor (Bergh *et al.* 2005).

The present review will focus on the role of thyroid hormone signalling via integrin $\alpha v \beta 3$ in tumour angiogenesis with an emphasis on tetrac in its function as angiogenesis inhibitor and discuss the implications for the management of cancer patients.

Thyroid hormones and cancer – clinical data

The earliest record of a possible relationship between thyroid hormone and cancer was composed by Beatson in 1896 – without knowing about the existence of hormones or what cancer actually was – when he treated breast cancer patients with thyroid extract as ‘powerful lymphatic stimulant’, noting, however, that ‘by itself thyroid extract seems to have little effect on the cancerous process’ (Beatson 1896). Since then, numerous studies, clinical and population-based, as well as experimental, have tried to shed light on the potential effects of thyroid hormone on both the risk of developing cancer and its effects on existing tumours. In contrast to Beatson’s observations, our current knowledge does suggest a connection between thyroid function and cancer, though there is no consensus on how this occurs and thus, 120 years later, the issue remains controversial (Table 1).

Thyroid hormone status and cancer survival

Several studies have examined the relationship between cancer survival and alterations in thyroid status. In prostate cancer patients, hyperthyroidism, in this case higher T3 levels, was associated with a higher clinical stage and a higher risk of recurrence (Lehrer *et al.* 2001). Hypothyroid lung cancer patients, in contrast, had an increased median survival and were older at disease onset (Hercbergs *et al.* 2010). Furthermore, invasive breast cancer incidence, and incidence of lymph node metastases, were found to be significantly lower with slower progression and smaller, more likely localised tumours in hypothyroid breast cancer patients as compared to euthyroid patients that were more likely to have metastatic disease (Cristofanilli *et al.* 2005, Hercbergs *et al.* 2010, Angelousi *et al.* 2017). However, another study found no relationship between thyroid function and breast cancer survival

(Jiskra *et al.* 2007). To complicate things further, hypothyroidism was found to be associated with a poorer overall and/or recurrence-free survival in patients with uveal melanoma and hepatocellular carcinoma (Oba *et al.* 2017, Zhang *et al.* 2018). Furthermore, low T3 syndrome, characterised by low serum T3 with low or normal free T4 and normal thyroid-stimulating hormone (TSH) levels, was associated with reduced progression-free survival and overall survival in diffuse large B cell lymphoma patients. Patients with synchronously low free T3 and free T4 had a worse outcome than did those with low free T3 alone (Gao *et al.* 2017). Free T4 levels were also shown to be positively correlated with improved breast cancer survival, while no association was found for free T3 (Brandt *et al.* 2016).

In addition to cancer mortality in patients with existing cancer, numerous epidemiological studies have tried to correlate the risk of developing cancer and the thyroid state. In this context, again both hyperthyroidism and hypothyroidism have been shown to influence cancer onset in both directions and thus no clear consensus exists. As this goes beyond the scope of the current review, please refer to recent studies and reviews for more information on the subject (Hellevik *et al.* 2009, Khan *et al.* 2016, Chan *et al.* 2017, Ortega-Olvera *et al.* 2018; reviewed in Hercbergs *et al.* 2010, Moeller & Führer 2013).

Thyroid dysfunctions as side effect of cancer treatment

Further evidence of thyroid hormone involvement in cancer progression has derived from the chance observation that cancer patients that develop hypothyroidism as unwanted side effect of treatment often show a more favourable outcome. In this context, a typical side effect of tyrosine kinase inhibitors is unintended hypothyroidism seen in 36–85% of patients (Buda-Nowak *et al.* 2017, Lechner *et al.* 2018b). This has been described for sunitinib and sorafenib in renal cell carcinoma patients, where a number of studies found a positive correlation of hypothyroidism with progression-free and/or overall survival compared to euthyroid patients, even with metastatic disease (Riesenbeck *et al.* 2011, Schmidinger *et al.* 2011, Baldazzi *et al.* 2012, Kust *et al.* 2014, Buda-Nowak *et al.* 2017). However, in another study, after T4 supplementation of all subclinically and overtly hypothyroid patients with metastatic disease treated with sunitinib, no correlation with survival and no difference between supplemented patients and naturally

Table 1 Effects of thyroid function on cancer outcomes.

Cancer type	Thyroid function and outcome	References
Breast	Hypothyroidism: slower progression, smaller, more localised tumours, fewer metastases	Cristofanilli <i>et al.</i> (2005)
Breast	Hypothyroidism: smaller tumours, lower stage	Hercbergs <i>et al.</i> (2010)
Breast	Hypothyroidism: lower incidence of metastases	Angelousi <i>et al.</i> (2017)
Breast	No prognostic significance of free T4 or TSH on relapse-free and overall survival	Jiskra <i>et al.</i> (2007)
Breast	Free T4 positively correlated with improved survival	Brandt <i>et al.</i> (2016)
Glioma	Chemically induced hypothyroidism: tumour regression, prolonged progression-free and overall survival	Hercbergs <i>et al.</i> (2003) , Ashur-Fabian <i>et al.</i> (2013)
Head and neck	Radiation-induced hypothyroidism: improved recurrence-free and overall survival	Nelson <i>et al.</i> (2006)
Hepatoma	Hypothyroidism: poorer recurrence-free and overall survival	Zhang <i>et al.</i> (2018)
Lung	Amiodarone-induced severe hypothyroidism: patient entered remission (single case report)	Hercbergs & Leith (1993)
Lung	Hypothyroidism: increased median survival	Hercbergs <i>et al.</i> (2010)
Lymphoma	Low T3 syndrome: reduced progression-free and overall survival; worse outcome when both free T3 and free T4 were low	Gao <i>et al.</i> (2017)
Melanoma	Interleukin-2-induced hypothyroidism: improved progression-free survival	Reid <i>et al.</i> (1991)
Prostate	Hyperthyroidism (high T3): higher clinical stage, higher risk of recurrence	Lehrer <i>et al.</i> (2001)
Renal	Interleukin-2-induced hypothyroidism: higher rate of remission, improved progression-free survival	Reid <i>et al.</i> (1991) , Weijl <i>et al.</i> (1993)
Renal	Tyrosine kinase-induced hypothyroidism: increased progression-free and/or overall survival	Riesenbeck <i>et al.</i> (2011) , Schmidinger <i>et al.</i> (2011) , Baldazzi <i>et al.</i> (2012) , Kust <i>et al.</i> (2014) , Buda-Nowak <i>et al.</i> (2017)
Renal	Tyrosine kinase-induced hypothyroidism: no effect on progression-free survival under thyroid hormone supplementation	Sabatier <i>et al.</i> (2012)
Uveal melanoma	Hypothyroidism: poorer survival	Oba <i>et al.</i> (2017)
Diverse	Induced euthyroid hypothyroxinaemia (low T4, normal T3): prolongation of survival in advanced cancers	Hercbergs <i>et al.</i> (2015)
Diverse	Tyrosine kinase-induced hypothyroidism: improved overall survival	Lechner <i>et al.</i> (2018a,b)

TSH, thyroid-stimulating hormone.

euthyroid patients was found ([Sabatier *et al.* 2012](#)). This suggested that thyroid hormone replacement may reduce the survival benefits gained from hypothyroidism ([Sabatier *et al.* 2012](#)). A recent retrospective study in patients with advanced non-thyroidal tumours of different entities has confirmed an overall survival benefit for those patients that developed hypothyroidism after tyrosine kinase inhibitor therapy. Here, however, thyroid hormone replacement therapy further improved overall survival as compared to those that did not receive replacement therapy ([Lechner *et al.* 2018a](#)).

Similarly, in a retrospective analysis of patients with head and neck squamous cell cancer, patients that developed hypothyroidism from radiation treatment had a significantly lower risk of death or recurrence ([Nelson *et al.* 2006](#)).

Studies from the 1990s using interleukin-2 treatment against renal cell cancer and malignant melanoma that can cause thyroiditis with subsequent hypothyroidism in some patients found less risk of death from progressive disease under hypothyroidism and partial/complete remission in the majority of hypothyroid patients ([Reid *et al.* 1991](#), [Weijl *et al.* 1993](#)).

In a single case report, treatment of a patient with metastatic non-small cell lung cancer with amiodarone HCl for cardiac arrhythmia caused severe amiodarone-induced hypothyroidism (myxoedema coma) with extremely low T4. The patient entered clinical remission, an extremely rare event in lung cancer, coincident with the severe thyroid hormone deficiency. Though the patient subsequently received thyroxine supplementation, no disease recurrence or progression was documented ([Hercbergs & Leith 1993](#)).

Induced hypothyroidism for cancer treatment

In syngeneic and xenograft rodent models of breast, liver, lung and prostate cancer, sarcoma and fibrosarcoma, the induction of hypothyroidism – induced chemically, by radioiodine ablation or by surgical thyroidectomy – led to a reduction in tumour growth in all models studied as compared to untreated controls (Shoemaker *et al.* 1976, Kumar *et al.* 1979, Mishkin *et al.* 1981, Theodossiou *et al.* 1999, Theodossiou & Schwarzenberger 2000, Martinez-Iglesias *et al.* 2009, Moeller & Führer 2013). Based on these findings, hypothyroidism has been deliberately induced in cancer patients in a few pioneering studies. And indeed, propylthiouracil (PTU)-induced mild hypothyroidism in glioma patients, including grade IV astrocytoma, was associated with tumour regression and a statistically significant prolongation of survival and time-to-progression (Herbergs *et al.* 2003, 2010). Likewise, after failing first-line standard treatment, a patient with inoperable grade IV glioblastoma multiforma of the optic chiasm was treated with PTU and thioamide to chemically induce hypothyroidism in conjunction with carboplatin chemotherapy leading to successful long-term tumour response, and prolonged overall survival (Ashur-Fabian *et al.* 2013). Interestingly, pharmacologic induction of a euthyroid hypothyroxinemic state under maintenance of normal circulating T3 levels and euthyroidism with substantial reduction of circulating T4 levels in advanced cancer patients with brain, ovary, lung, pancreas and salivary gland tumours, mesothelioma and soft-tissue sarcoma was associated with a significant prolongation of actual versus expected survival (Herbergs *et al.* 2015).

Identifying an association between thyroid function and cancer progression or cancer risk is not straightforward, which probably underlies the high variance in study outcomes reported. For one, causality cannot be easily inferred, that is thyroid hormone and/or TSH levels, often used as sole indicator of thyroid function, may be influenced by the cancer itself and not necessarily vice versa (Brandt *et al.* 2016, Chan *et al.* 2017). Further, other co-morbidities and lifestyle choices may affect both thyroid function and cancer progression at the same time. Age, obesity, hypertension, diabetes, smoking, alcohol consumption and dyslipidaemia have been associated with cancer mortality and incidence, and have been reported to alter thyroid hormone and/or TSH levels (Belin *et al.* 2004, Jorde & Sundsfjord 2006, Asvold *et al.* 2007, Mehran *et al.* 2012, Yeap *et al.* 2012, Tseng *et al.* 2015). In the case of sex hormone-dependent cancers,

such as breast, ovarian and prostate cancer, changes in thyroid hormone concentrations may affect circulating sex hormone concentrations with different effects on tumour subtypes depending on receptor status (Saraiva *et al.* 2005, Chan *et al.* 2017). In this context, the menopausal status of women is a further critical factor. Not all studies are corrected for these confounders. Variation in inclusion criteria, for example whether subclinical thyroid hormone disorders or patients on thyroid-related medications were included, as well as which and how often thyroid metabolites were measured to determine thyroid function, further confound comparability. In addition, studies are often based on small case numbers and consequently lack sufficient statistical power to reliably assess potential associations between thyroid function and cancer. At the level of the tumour itself, the ability to react to thyroid hormone depends on the expression of different receptors (see below). Furthermore, the local, tissue-specific thyroid hormone status in the tumour environment may differ from the systemic status. A local hypothyroid state in the liver has, for instance, been proposed to drive hepatocellular carcinoma development and progression (Perra *et al.* 2016).

In conclusion, a lot of effort has been put into the study of the role of thyroid hormones both in the aetiology and progression of cancer. So far, data from clinical and epidemiological studies remain highly inconsistent. Identifying those cancer patients that would profit from manipulation of their thyroid hormone levels remains an important open question.

Thyroid hormone signalling

The main secretory product of the thyroid gland is T4. Conversion to T3, conventionally acknowledged as the bioactive iodothyronine, occurs mainly in peripheral tissues by deiodinases. Canonical thyroid hormone effects are then exerted through binding of T3 to thyroid hormone receptor (TR) isoforms that are localised in the nucleus and act as ligand-dependent transcription factors (Cheng *et al.* 2010, Moeller & Führer 2013). In this ‘classical’ or ‘genomic’ pathway, T4 functions as pro-hormone for T3, as most of the thyroid hormone bound to TRs is T3 (Sinha & Yen 2000, Brent 2012). Besides effects involving T3 binding to nuclear hormone receptors, several so-called ‘non-genomic’ pathways have been identified, where initiation occurs in the cytoplasm, the mitochondria, or, as in the focus of this review, at the plasma membrane (Davis *et al.* 2018).

Cell surface signalling

An early indicator of the potential existence of a membrane-based TR was the observation that ^{131}I -labelled T3 and T4 bind to the cell membrane of enucleated erythrocytes, providing the basis for an *in vitro* method to measure free hormone concentrations in patients and thus evaluate their thyroid function (Freedberg *et al.* 1957, Hamolsky *et al.* 1959, Schwartz *et al.* 1968). In an attempt to elucidate the molecular structure essential for binding of thyroid hormone to membranes, Singh *et al.* observed that the T4 derivative tetrac displaces ^{131}I -T3 and ^{131}I -T4 from the membrane (Singh *et al.* 1976). That the binding site may function as a hormone receptor in the intact cell became apparent when ion pumps such as the Ca^{2+} ATPase were found to be rapidly activated by thyroid hormone, both in enucleated and nucleated cells, via activation of protein kinase signal transduction pathways (reviewed in Lin *et al.* 2012, Davis *et al.* 2018). Subsequently, both T3 and T4 were found to effectively activate the mitogen-activated protein kinase (MAPK; extracellular signal-regulated kinases 1/2 (ERK1/2)) pathway in cells that lack TR expression. T4 bound to an agarose bead, restricting it to the extracellular space, reproduced this effect, demonstrating that it had to be plasma membrane-initiated. Tetrac, in contrast, inhibited the kinase activation (Lin *et al.* 1999a). Despite growing evidence for the existence of a cell surface TR, it was not until 2005 that Paul Davis' laboratory identified the elusive receptor as integrin $\alpha\text{v}\beta 3$ (Bergh *et al.* 2005).

Thyroid hormone receptor on integrin $\alpha\text{v}\beta 3$

Integrins are heterodimeric cell surface receptors involved in cell-cell and cell-extracellular matrix protein interactions, and have emerged as major regulators of the activity of various cell signalling pathways. Though integrins themselves are not oncogenic, their expression by tumour and tumour stroma-associated cells critically contributes to tumour progression and metastasis (Desgrosellier & Cheresch 2010). Ligand-binding to the extracellular domain is translated into downstream signalling events via outside-in signalling inside the cell, usually engaging signal-transducing kinase cascades (Xiong *et al.* 2001). Integrin signalling is typically transduced into cell motility, adhesion, division and angiogenesis via direct effects on the cytoskeleton and modulation of gene transcription. Furthermore, crosstalk between integrins and neighbouring growth factor and cytokine receptors, affecting their expression,

ligand affinity and signalling, seems to be required for many of their tumour-promoting effects (Desgrosellier & Cheresch 2010).

That the integrin $\alpha\text{v}\beta 3$ is a mediator of tumour-related angiogenesis is now a well-established concept that was first described in the 1990s (Brooks *et al.* 1994). Tumour-associated vessels, unlike quiescent endothelium, express the integrin, and its expression on tumour cells is correlated with disease progression (Desgrosellier & Cheresch 2010). Its prototypic ligand is the extracellular matrix glycoprotein vitronectin that binds to the integrin's extracellular domain via an RGD (arginine-glycine-aspartic acid) sequence (Ruoslahti & Pierschbacher 1987). The thyroid hormone-binding site on integrin $\alpha\text{v}\beta 3$ is located near this RGD recognition site and comprises two distinct domains that are related neither structurally nor functionally to TRs (Cody *et al.* 2007, Lin *et al.* 2009). The binding site S1 exclusively binds T3 and causes activation of the phosphoinositide 3-kinase (PI3K) cascade, while S2 binds both T3 and T4 and results in MAPK (ERK1/2) pathway activation (Lin *et al.* 2009). Thus, in contrast to the TR/thyroid hormone axis, both T4 and T3 act as active hormones at the integrin receptor. In fact, as the affinity for T4 is far higher than that for T3, and lies in the physiologic concentration range that is comparable to the T3 affinity for nuclear receptors, T4 may well be the main hormone signalling at the integrin, thus transcending its role of pro-hormone for T3 (Davis *et al.* 2013b). Tetrac binds to both thyroid hormone-binding sites on the integrin and is a specific inhibitor of integrin-mediated thyroid hormone action (Wu *et al.* 2005, Mousa *et al.* 2006). Within the cell, in contrast, tetrac has low-grade thyromimetic activity, both by itself, as well as through its conversion to triiodothyroacetic acid (triac) (Burger *et al.* 1979, Moreno *et al.* 2008). By covalently binding tetrac to an approximately 200 nm, biodegradable nanoparticle (Nanotetrac), it is excluded from the cell interior and thus acts exclusively at the integrin (Yalcin *et al.* 2009). The genes that are modulated from the extracellular domain of integrin $\alpha\text{v}\beta 3$ by thyroid hormone relate to cancer cell proliferation, apoptosis, chemo/radioresistance and angiogenesis (Davis *et al.* 2014a).

Thyroid hormones and angiogenesis

Angiogenesis, the formation of new blood vessels from the existing vasculature, is a highly regulated process, temporally and spatially controlled by a balance between pro- and anti-angiogenic factors (Pinto *et al.* 2011,

Bronckaers *et al.* 2014). In response to specific stimuli, including hypoxia and inflammation, the angiogenic switch occurs in the quiescent endothelium, initiating coordinated endothelial cell proliferation, invasion, migration and ultimately, tube formation and maturation. Angiogenesis is closely linked to tumour progression, both sustaining tumour growth by providing oxygen and other nutrients, and facilitating metastasis. While physiological angiogenesis in the context of development, tissue growth and wound-healing is tightly regulated and self-limiting, cancer-associated angiogenesis, though in principle regulated by the same pathways, is usually persistent and abnormal (Avraamides *et al.* 2008).

Angiogenesis is orchestrated by local release of vascular growth factors and their discrete receptors on the cell surface that then transduce specific signals into angiogenesis-related events. The vascular endothelial growth factor (VEGF) is the archetypal stimulator of angiogenesis, both under normal physiologic conditions and in the tumour environment. VEGF initiates the angiogenic process by activating endothelial cells, fostering their migration and proliferation, and mediating vascular permeability (Somanath *et al.* 2009). Other major pro-angiogenic vascular growth factors include fibroblast growth factor 2 (FGF2, also known as basic fibroblast growth factor; bFGF), platelet-derived growth factor (PDGF) and the angiopoietins. The angiogenic process is additionally supported by contributions from less specific factors, such as the epidermal growth factor (EGF), transforming growth factors (TGF)- α and - β , and cytokines such as interleukins -1 (IL-1), -6 (IL-6) and -8 (IL-8) and their respective receptors. Endogenous inhibitors of angiogenesis include thrombospondin 1, angiostatin and endostatin (Avraamides *et al.* 2008, Bronckaers *et al.* 2014). These angiogenesis-relevant factors are produced by tumour cells, the endothelial cells themselves and by cells of the tumour stroma such as cancer-associated fibroblasts, pericytes and infiltrating immune cells (Ellis 2004, Bronckaers *et al.* 2014).

Discovery of the pro-angiogenic nature of thyroid hormones

Chilian *et al.* made the initial observation of a pro-angiogenic effect of thyroid hormones when they discovered that induction of myocardial hypertrophy in the rat by thyroxine leads to a higher capillary density *before* hypertrophy sets in, that is that angiogenesis is not driven by tissue enlargement of the heart *per se* (Chilian *et al.* 1985, Tomanek & Busch 1998).

However, it took another two decades until the underlying mechanisms were systematically addressed using the chick chorioallantoic membrane (CAM) angiogenesis model, not only establishing that iodothyronines are angiogenesis modulators, but also leading to the discovery of the thyroid hormone receptor on integrin $\alpha\text{v}\beta 3$. The group around Paul Davis could show that (1) both T3 and T4 are pro-angiogenic, T4 at physiological levels and to a greater extent; (2) the blocking of conversion of T4 to T3 with the deiodinase inhibitor PTU did not abrogate the pro-angiogenic effect of T4; (3) agarose-linked T4 reproduced the angiogenic effects; (4) while tetrac alone had no effect on angiogenesis, it did block the effects of T4 and T4-agarose; (5) the addition of an antibody against FGF2 abrogated angiogenesis stimulated by T4, suggesting that, at least in part, the pro-angiogenic effect of T4 is mediated by increased FGF2 expression; and (6) inhibition of the MAPK (ERK1/2) pathway completely blocked pro-angiogenic thyroid hormone action. They could further show that T4 could rapidly (within 6 h) induce FGF2 mRNA expression in endothelial cells and that both T3 and T4 were able to foster FGF2 secretion from endothelial cells in a MAPK (ERK1/2)-dependent manner (Davis *et al.* 2004).

Iodothyronines have since emerged as non-classical pro-angiogenic modulators that induce neovascularisation via integrin $\alpha\text{v}\beta 3$ -based pathways in a variety of settings, including the heart, ischaemic striated muscle and the tumour microenvironment (Luidens *et al.* 2010). Acting at integrin $\alpha\text{v}\beta 3$, tetrac antagonises stimulation of angiogenesis by thyroid hormone (Table 2).

Inhibition of pro-angiogenic thyroid hormone effects by tetrac

Tetrac was shown to inhibit the potentiation by T4 of EGF- and TGF- α -induced signal transduction via MAPK (ERK1/2) in HeLa cells that lack nuclear TRs (Lin *et al.* 1999b, Shih *et al.* 2004). Both EGF and TGF- α are EGF receptor (EGFR) ligands that are known to induce VEGF expression (Ellis 2004). Similarly, phosphorylation of protein kinase D and phosphorylation and cytoplasmic accumulation of histone deacetylase 5, implicated in regulation of gene transcription and angiogenesis downstream of VEGF signalling, were stimulated by T4, leading to increased FGF2 expression and decreased by additional treatment with tetrac in endothelial cells (Ha *et al.* 2008, Liu *et al.* 2014). Furthermore, thyroid hormone-enhanced matrix metalloproteinase 9 transcription and activity were subject to inhibition by tetrac, as was the activity of PDGF

Table 2 Angiogenesis-relevant targets of thyroid hormones and tetrac.

Target	Effect	Model	References
Tube formation	↑ T3, T4 ↓ tetrac	Endothelial cells, CAM	Mousa <i>et al.</i> (2006, 2008), Balzan <i>et al.</i> (2013), Liu <i>et al.</i> (2014)
Endothelial cell migration	↑ T3, T4 ↓ tetrac	Endothelial cells	Balzan <i>et al.</i> (2013), Liu <i>et al.</i> (2014)
Endothelial cell proliferation	↑ T3, T4 ↓ tetrac	Endothelial cells	Balzan <i>et al.</i> (2013)
FGF2 expression and secretion	↑ T3, T4 ↓ tetrac	Endothelial cells, CAM	Davis <i>et al.</i> (2004), Liu <i>et al.</i> (2014)
VEGF expression	↑ T3, T4 ↓ tetrac	Endothelial cells	Balzan <i>et al.</i> (2013)
Angiopoietin-1 expression	↑ T3, T4 ↓ tetrac	Endothelial cells	Balzan <i>et al.</i> (2013)
MMP9 expression and activation	↑ T3, T4 ↓ tetrac	Cancer cells (multiple myeloma)	Cohen <i>et al.</i> (2014)
HIF-1 α transcription	↑ T4 ↓ tetrac*	Cancer cells (lung and ovarian)	Lin <i>et al.</i> (2013), Davis <i>et al.</i> (2015)
EGF- and TGF- α -induced pro-angiogenic signalling	↑ T4 ↓ tetrac	Cancer cells (cervical)	Shih <i>et al.</i> (2004)
Pericyte marker expression	↑ T3, T4 ↓ tetrac	Mesenchymal stem cells/ hepatoma	Schmohl <i>et al.</i> (2015)
Pro-angiogenic (growth) factor expression and secretion	↑ T3, T4 ↓ tetrac	Mesenchymal stem cells/ hepatoma	Schmohl <i>et al.</i> (2015), Schmohl <i>et al.</i> unpublished observations**
Hypoxia response and VEGF signalling	↑ T3, T4 ↓ tetrac	Mesenchymal stem cells/ hepatoma	Schmohl <i>et al.</i> unpublished observations**
Tumour recruitment of angiogenesis-promoting cells	↑ T3, T4 ↓ tetrac	Mesenchymal stem cells/ hepatoma	Schmohl <i>et al.</i> (2015)

*proposed effect. **Schmohl KA, Mueller AM, Dohmann M, Spellerberg R, Urnauer S, Schwenk N, Ziegler SI, Nelson PJ & Spitzweg C, unpublished observations.

CAM: chorioallantoic membrane; EGF: epidermal growth factor; FGF2: fibroblast growth factor 2; HIF-1 α : hypoxia-inducible factor 1 α ; MMP9: matrix metalloproteinase 9; TGF- α : transforming growth factor α ; VEGF: vascular endothelial growth factor.

(Cohen *et al.* 2014, Davis *et al.* 2014b). Matrix metalloproteinases promote angiogenesis by preparing the vascular bed, that is facilitating endothelial cell detachment and paving the way for the advancing vascular sprout, and through sequestration and activation of angiogenic mediators such as VEGF from the extracellular matrix (Davis *et al.* 2015, Deryugina & Quigley 2015). In addition, thyroid hormone stimulates endothelial cell proliferation, migration and vascular tube formation, all important prerequisites for angiogenesis – activities shown to be inhibited by tetrac (Mousa *et al.* 2006, Balzan *et al.* 2013, Liu *et al.* 2014, Mousa *et al.* 2014).

Effects on the hypoxia response

Hypoxia, that is low oxygen tension, occurs in rapidly growing and progressively more poorly vascularised tumours. This leads to increased expression and stabilisation of hypoxia-inducible factor-1 α (HIF-1 α) and subsequent heterodimerisation with HIF-1 β to form HIF-1. This master regulator of the adaptive response of tissues to hypoxia fosters the transcription of a host of genes relevant

to tumour progression and angiogenesis, including VEGF (Liu *et al.* 2009, Semenza 2010). HIF-1 α transcription is sensitive to thyroid hormone: As shown in human non-small cell lung cancer and ovarian carcinoma cells, T4, but not T3, induces internalisation of integrin α v β 3, resulting in a complex formed between the α v monomer and MAPK (ERK1/2), leading to nuclear import (Lin *et al.* 2013). In the nucleus, α v acts as a transcriptional co-activator and helps to drive the transcription of HIF-1 α . Tetrac and Nanotetrac inhibit integrin internalisation, and thus, can block this angiogenic effect of thyroid hormone (Lin *et al.* 2011, Davis *et al.* 2014a, 2015). Interestingly, the α v gene (*ITGAV*) itself, in turn, contains hypoxia response elements in its promoter region and is hence a HIF-1 target gene (Nieberler *et al.* 2017). The HIF-1 α -related pro-angiogenic activity of thyroid hormone is thought to be especially relevant in the context of a hypoxic tumour setting. Furthermore, T3 has been reported to induce HIF-1 α expression by a PI3K-dependent mechanism (Moeller *et al.* 2005, Moeller *et al.* 2006). Whether this effect is initiated at a cytoplasmically located TR, or at the cell surface at integrin α v β 3, or both, remains to be elucidated (Luidens *et al.* 2010).

Thus, tetrac, functioning as a thyroid hormone antagonist at integrin $\alpha v \beta 3$, interferes with numerous pro-angiogenic activities of thyroid hormones, which ultimately leads to decreased vessel formation.

Agonist-independent effects of tetrac on angiogenesis

Tetrac was initially thought to exert its anti-angiogenic actions only by disrupting interaction of thyroid hormone with the integrin receptor. However, it was subsequently found to block the pro-angiogenic activity of VEGF and FGF2 in the *absence* of agonist hormone (Table 3).

Effects on transcription and activity of vascular growth factors and their receptors

Quantitative real-time PCR and microarray studies in different human cancer cells *in vitro* have confirmed anti-angiogenic effects of tetrac on gene transcription: Tetrac and Nanotetrac were shown to significantly decrease the transcription of pro-angiogenic VEGF in human medullary thyroid cancer cells (Yalcin *et al.* 2010b). Nanotetrac, but not unmodified tetrac, decreased the transcription of EGFR in human breast cancer and pancreatic carcinoma cells (Glinskii *et al.* 2009, Yalcin *et al.* 2013). This observation is significant in that the abundance and activity of EGFR is abnormally elevated in most solid tumours and is

Table 3 Agonist thyroid hormone-independent angiogenesis-relevant targets of tetrac and Nanotetrac.

Target	Effect	Model	References
Tube formation	↓ tetrac	Endothelial cells	Mousa <i>et al.</i> (2008), Yoshida <i>et al.</i> (2012), Balzan <i>et al.</i> (2013)
Endothelial cell migration	↓ tetrac	Endothelial cells	Yoshida <i>et al.</i> (2012)
Endothelial cell proliferation	↓ tetrac	Endothelial cells	Yoshida <i>et al.</i> (2012), Balzan <i>et al.</i> (2013)
VEGF-induced angiogenesis	↓ tetrac	CAM	Mousa <i>et al.</i> (2008)
FGF2-induced angiogenesis	↓ tetrac ↓ Nanotetrac	CAM, mouse Matrigel plug assay	Mousa <i>et al.</i> (2008), Bharali <i>et al.</i> (2013)
VEGF transcription	↓ tetrac ↓ Nanotetrac	Cancer cells (thyroid)	Yalcin <i>et al.</i> (2010b)
EGFR transcription	↓ Nanotetrac	Cancer cells (breast and pancreas)	Glinskii <i>et al.</i> (2009), Yalcin <i>et al.</i> (2013)
Angiopoietin-like 1, 2 and 3 transcription	↓ tetrac	Endothelial cells	Mousa <i>et al.</i> (2008)
MMP15, 19 and 24 transcription	↓ tetrac	Endothelial cells	Mousa <i>et al.</i> (2008)
MMP inhibitor TIMP3 levels	↑ tetrac	Endothelial cells	Mousa <i>et al.</i> (2008)
Pro-angiogenic cytokine and receptor transcription	↓ Nanotetrac	Cancer cells (breast and thyroid)	Davis <i>et al.</i> (2013a, 2016)
Angiostatic cytokine CXCL10 transcription	↑ Nanotetrac	Cancer cells (breast and thyroid)	Davis <i>et al.</i> (2013a, 2016)
Angiopoietin-2 levels	↓ tetrac	Endothelial cells	Mousa <i>et al.</i> (2008)
MMP9 transcription and activity	↓ tetrac	Cancer cells (multiple myeloma)	Cohen <i>et al.</i> (2014)
Thrombospondin 1 transcription	↑ tetrac ↑ Nanotetrac	Cancer cells (breast, oral, pancreas, thyroid)	Glinskii <i>et al.</i> (2009), Yalcin <i>et al.</i> (2010b, 2013), Lin <i>et al.</i> (2018)
Vascular growth factor activity via receptor crosstalk inhibition	↓ tetrac	Diverse	Davis <i>et al.</i> (2013b, 2015), Incerpi <i>et al.</i> (2014)
Pro-angiogenic activity of LPS, bradykinin and angiotensin	↓ Nanotetrac	CAM	Mousa <i>et al.</i> (2014)
Pro-angiogenic miR-21 levels	↓ Nanotetrac	Cancer cells (breast)	Davis <i>et al.</i> (2014b)
Angiostatic miR-15A levels	↑ Nanotetrac	Cancer cells (breast)	Davis <i>et al.</i> (2014b)
Tumoural haemoglobin content	↓ tetrac ↓ Nanotetrac	Cancer cell xenografts in mice (glioblastoma, lung, pancreas, renal, thyroid)	Yalcin <i>et al.</i> (2009, 2010a,b, 2013), Mousa <i>et al.</i> (2012)
Tumoural haemoglobin content and VEGF levels	↓ tetrac	Murine Lewis lung carcinoma	Carmona-Cortes <i>et al.</i> (2014)
Tumour vascularity	↓ Nanotetrac	Cancer cell xenografts in mice (glioblastoma)	Sudha <i>et al.</i> (2017a)
Tumoural NF- κ B activity	↓ Nanotetrac	Cancer cell xenografts in mice (urinary bladder)	Sudha <i>et al.</i> (2017b)

CAM, chorioallantoic membrane; EGFR, epidermal growth factor receptor; FGF2, fibroblast growth factor 2; LPS, lipopolysaccharide; miR, microRNA; MMP, matrix metalloproteinase; NF- κ B, nuclear factor kappa B; TIMP3, tissue inhibitor of metalloproteinase 3; VEGF, vascular endothelial growth factor.

associated with cancer progression and poor prognosis. Though not a specific angiogenesis-related growth factor, stimulation or inhibition of EGFR has been shown to have significant consequences for tumour angiogenesis (van Cruijsen *et al.* 2005).

In addition to the direct effects on vascular growth factor/vascular growth factor receptor gene transcription, tetrac was also found to modulate their activity. In detail, tetrac was shown to block VEGF-induced human dermal microvascular endothelial cell tube formation *in vitro* and the pro-angiogenic activity of FGF2, VEGF and T3 in the CAM assay (Mousa *et al.* 2008). While unmodified tetrac inhibited FGF2-induced angiogenesis by 60%, nanoparticulate tetrac achieved a near-complete inhibition in the CAM assay (Bharali *et al.* 2013). In a subcutaneous mouse Matrigel plug assay, tetrac treatment resulted in a significant inhibition of FGF2-induced angiogenesis, as measured by haemoglobin content (Mousa *et al.* 2008). In human retinal endothelial cells, tetrac was reported to decrease VEGF- and erythropoietin-triggered proliferation, inhibit both basal and VEGF-induced migration, and VEGF-induced tube formation *in vitro* (Yoshida *et al.* 2012). Similarly, Balzan *et al.* reported that tetrac at high concentrations (5 μ M) inhibited basal and VEGF-induced human cardiac microvascular endothelial cell proliferation and tube formation. In a wound-healing assay, tetrac did not influence basal cell migration, but did inhibit enhanced migration by VEGF (Balzan *et al.* 2013). Tetrac has further been shown to block the pro-angiogenic activities of EGFR and PDGFR, and is hypothesised to inhibit the actions of the insulin-like growth factor 1 receptor (Davis *et al.* 2013c, 2015, Incerpi *et al.* 2014).

The basis for the ability of tetrac to disrupt vascular growth factor signalling has been hypothesised to lie in non-transcriptional modulation of the function of vascular growth factor receptors adjacent to $\alpha v\beta 3$, by interrupting crosstalk between the integrin and growth factor receptors (Davis *et al.* 2015). Activation of intracellular signalling cascades in endothelial cells by growth factors is amplified when the cells are attached to the extracellular matrix, indicating a synergy between integrin and growth factor signalling. Indeed, integrin $\alpha v\beta 3$ co-clusters and directly interacts with VEGFR, FGFR and PDGFR (Somanath *et al.* 2009). Disruption of the interaction between integrin $\alpha v\beta 3$ and VEGFR or FGFR results in diminished activation by their respective ligands and impairs integrin activation (Somanath *et al.* 2009). The RGD recognition pocket plays an essential role in facilitating interactions of the integrin with extracellular matrix proteins, and pro-angiogenic

activities of VEGF and FGF2 are blocked by RGD peptides (Yalcin *et al.* 2009). Tetrac binds to the integrin near the RGD recognition site and may either directly occlude the binding sites for vascular growth factors or lead to conformational changes in the highly plastic integrin, resulting in allosteric inhibition of interaction with vascular growth factors/receptors at the RGD site (Mousa *et al.* 2008).

Thus, depending on the growth factor, the biologic actions of tetrac can involve gene transcription, growth factor release or inhibitory crosstalk between the integrin and vascular growth factor receptors clustered with the integrin in the plasma membrane.

Effects on cytokines and their receptors

In addition to vascular growth factors, a number of angiogenesis-relevant interleukins as well as chemokines and their receptors are subject to transcriptional modulation by tetrac in human cancer cell lines. Nanotetrac was shown to reduce the abundance of IL-1 α and β mRNA in human breast cancer cells, as determined by microarray gene expression profiling (Davis *et al.* 2013a). The IL-1 family has been shown to interact with VEGF to initiate and maintain tumour angiogenesis (Voronov *et al.* 2014). IL-6, a stimulator of a plethora of pro-angiogenic molecules, including VEGF, FGF2 and HIF-1 α , and of endothelial cell migration and proliferation, can also be downregulated by Nanotetrac treatment (Davis *et al.* 2013a, Middleton *et al.* 2014). By contrast, the expression of IL-11, a member of the IL-6 family of interleukins, was shown to be increased (Davis *et al.* 2013a). Though not as conclusively explored as IL-6, IL-11 has been shown to protect microvascular endothelium from inflammation and injury, and to act as a growth factor for haematopoietic stem cells and megakaryocyte progenitor cells (Kirkiles-Smith *et al.* 2004, Hercbergs *et al.* 2012, Johnstone *et al.* 2015). To what extent these observations influence tumour angiogenesis remains to be elucidated.

Nanotetrac was further shown by microarray analysis to decrease the steady-state mRNA levels of the chemokines CCL20, CXCL2, CXCL3 and CX3CL1 in human breast and thyroid cancer cells in a highly correlated pattern (Davis *et al.* 2013a, 2016). All of these chemokines have been shown to be pro-angiogenic in diverse contexts, including cancer angiogenesis (Mehrad *et al.* 2007, Ryu *et al.* 2008, Keeley *et al.* 2010, Owen & Mohamadzadeh 2013, Li *et al.* 2016). CXCL10 transcription, in contrast, is stimulated by Nanotetrac in both cell lines (Davis *et al.* 2013a, 2016).

As CXCL10 has angiostatic properties via its receptor CXCR3, this observation fits with the overall anti-angiogenic effects of Nanotetrac at the integrin (Mehrad *et al.* 2007, Raman *et al.* 2011). Further, the chemokine receptor genes *CCR1*, *CXCR4* and *CX3CR4* are transcriptionally modulated by treatment with Nanotetrac. Transcription of *CCR1* was shown to be downregulated by Nanotetrac in breast cancer cells and, in contrast, slightly upregulated in thyroid cancer cells (Davis *et al.* 2016). *CCR1* via its ligands *CCL3* and *CCL5/RANTES* is generally thought to be pro-angiogenic, by both directly inducing tumour angiogenesis and initiating infiltration of pro-angiogenic macrophages (Marone & Granata 2014). Surprisingly, the transcription of *CXCR4* was increased by Nanotetrac (Davis *et al.* 2016). Its principal ligand is *CXCL12* and *CXCL12/CXCR4* signalling is known to stimulate tumour angiogenesis (Meng *et al.* 2018). Furthermore, transcription of *CX3CR1* is decreased by Nanotetrac (Davis *et al.* 2016). As detailed above, transcription of its ligand *CX3CL1* is also affected by Nanotetrac. *CX3CL1/CX3CR1* signalling induces angiogenesis via multiple pathways, including effects on *HIF-1 α* and *VEGF*, and is thought to be important for the structural integrity of vessels (Owen & Mohamadzadeh 2013, Szukiewicz *et al.* 2013). Interestingly, this ligand/receptor pair is proposed to form a ternary complex with integrin $\alpha\beta 3$, which leads to activation of the integrin (Goda *et al.* 2000, Davis *et al.* 2016). The integrin can also be directly, that is *CX3CR1*-independently, activated by *CX3CL1* by its binding to a site near the TR on the integrin (Fujita *et al.* 2014, Davis *et al.* 2016). This close proximity suggests that the binding sites may interact and *CX3CL1* binding may be influenced by thyroid hormone and tetrac (Davis *et al.* 2016). Moreover, as *CX3CL1* is known to undergo cell internalisation and thyroid hormone has been shown to drive internalisation of integrin $\alpha\beta 3$, Davis *et al.* have hypothesised that cellular uptake of *CX3CL1* may be modulated by thyroid hormone binding to its site on the integrin (Lin *et al.* 2013, Davis *et al.* 2016). This interference of tetrac with the transcription of chemokine/chemokine receptor genes constitutes an important aspect of its anti-tumour activity. Aside from angiogenesis, chemokine signalling is also fundamental for intra- and intercellular communication in the tumour microenvironment.

Effects on transcription of other angiogenesis-relevant genes

Tetrac and Nanotetrac were shown to significantly increase the transcription of thrombospondin 1, an endogenous inhibitor of angiogenesis commonly suppressed in

cancer cells, including human medullary thyroid, breast, pancreas and oral cancer cells (Glinskii *et al.* 2009, Yalcin *et al.* 2010b, Yalcin *et al.* 2013, Lin *et al.* 2018). Microarray analysis of *VEGF*-stimulated human dermal microvascular endothelial cells has suggested that tetrac dose-dependently decreases the abundance of angiopoietin-2, but not angiopoietin-1. The latter contributes to the maturation of blood vessels and maintenance of the resting endothelial state, while its antagonist angiopoietin-2 enhances vascular permeability and destabilises the quiescent endothelium in preparation of vascular network expansion (Mousa *et al.* 2008, Somanath *et al.* 2009). Furthermore, the expression of mRNA for angiopoietin-like 1, 2 and 3, proposed pro-angiogenic factors, was subject to inhibition by tetrac, as were the mRNAs of three different matrix metalloproteinases (15, 19 and 24) (Hato *et al.* 2008, Mousa *et al.* 2008). The abundance of tissue inhibitor of metalloproteinase 3, as the name suggests, a potent matrix metalloproteinase inhibitor, in contrast, was increased (Mousa *et al.* 2008). Similarly, in human multiple myeloma cell lines, tetrac inhibited matrix metalloproteinase 9 mRNA expression. Conditioned medium from these cells or from primary cells obtained from bone marrow aspirates from multiple myeloma patients treated with either tetrac or Nanotetrac had significantly decreased levels of matrix metalloproteinase 9 activity as shown by gelatin zymography (Cohen *et al.* 2014). Moreover, in the CAM assay, Nanotetrac inhibited the activity of lipopolysaccharide (LPS) and endogenous pro-angiogenic factors bradykinin and angiotensin II (Mousa *et al.* 2014). The underlying mechanisms are not known, though for bradykinin disruption of crosstalk between the integrin and *VEGFR* and/or *FGFR* by Nanotetrac has been proposed as the underlying mechanism (Davis *et al.* 2015).

On a slightly different note, Nanotetrac was shown to influence the cellular abundance of certain microRNAs (miRs), small non-coding RNAs that regulate gene transcription: anti-angiogenic microRNA miR-15A is increased and pro-angiogenic miR-21 decreased in human breast cancer cells upon treatment with Nanotetrac (Davis *et al.* 2014a).

Effects of tetrac on tumour angiogenesis *in vivo*

Both tetrac and its nanoparticulate formulation significantly reduced tumour angiogenesis as assessed by haemoglobin content both in the CAM assay and mouse xenografts implanted with human renal cell carcinoma, follicular thyroid carcinoma, medullary thyroid carcinoma, non-small cell lung cancer, pancreatic cancer and glioblastoma cells (Yalcin *et al.* 2009,

2010a,b, 2013, Mousa *et al.* 2012, Davis *et al.* 2015). In a subcutaneous murine Lewis lung carcinoma model, tetrac significantly reduced haemoglobin content and VEGF protein levels in tumour tissue compared to untreated controls, and restored normal VEGF levels in tumours of T4-treated animals (Carmona-Cortés *et al.* 2014). Nanotetrac, when injected intratumourally at the time of implant or after 10 days of daily subcutaneous injections, was further shown to decrease the vascularity of subcutaneous human glioblastoma xenografts in mice by 95% as determined by histopathological analysis, without inducing haemorrhage (Sudha *et al.* 2017a). This study quite impressively demonstrates that tetrac not only inhibits neo-angiogenesis but also seems to cause de-vascularisation. In addition, Nanotetrac was shown to suppress the activity of NF- κ B (nuclear factor kappa B) in human urinary bladder cancer xenografts in mice (Sudha *et al.* 2017b). This rapid response transcription factor is involved in stress response, is linked to cancer progression and is known to activate the transcription of pro-angiogenic mediators such as VEGF and IL-8, both directly and indirectly via activation of the hypoxia response pathway (Xie *et al.* 2010, Del Prete *et al.* 2011).

Hence, tetrac – independent of agonist thyroid hormone – affects the transcription and activity of various vascular growth factors, cytokines and other angiogenesis mediators, including miRs, as well as their respective receptors, cumulatively leading to reduced angiogenesis, an effect that is reflected by reduced angiogenesis in rodent tumour models.

Effects on tumour stroma-associated mesenchymal stem cells

The main body of evidence for integrin-mediated thyroid hormone effects in the context of cancer pertains to tumour cells and tumour-associated endothelial cells. However, mesenchymal stem cells, important progenitors to cell subtypes that comprise the tumour's fibrovascular network, are also integrin α v β 3-positive and are subject to modulation by thyroid hormone and tetrac (Table 2) (Schmohl *et al.* 2015; Schmohl KA, Mueller AM, Dohmann M, Spellerberg R, Urnauer S, Schwenk N, Ziegler SI, Nelson PJ & Spitzweg C, unpublished observations).

Mesenchymal stem cells and angiogenesis

Mesenchymal stem cells are multipotent non-haematopoietic progenitor cells that selectively home to sites of tissue injury and contribute to tissue remodelling

through differentiation and secretion of pro-angiogenic factors (Dwyer & Kerin 2010). They play multifaceted roles in promoting angiogenesis and are involved in all stages, including the early steps of endothelial cell recruitment, but also in the maturation of newly formed vessels (Cuiffo & Karnoub 2012, Bronckaers *et al.* 2014). In addition to the differentiation of mesenchymal stem cells into blood vessel-stabilising pericytes, their secretome contains a host of angiogenic factors that have been shown to alter endothelial cell behaviour.

Based on similarities between the wound-healing process and tumour stroma formation, mesenchymal stem cells are actively recruited to growing tumour stroma (Dvorak 1986, Knoop *et al.* 2011, 2015, Müller *et al.* 2016, Schug *et al.* 2019). Upon homing, they differentiate into cancer-associated fibroblast/pericyte-like cells that are characterised by the expression of pro-angiogenic factors and tumour-promoting growth factors (Mishra *et al.* 2008, Spaeth *et al.* 2009, Kidd *et al.* 2012, Barcellos-de-Souza *et al.* 2013, Schmohl *et al.* 2015; Schmohl KA, Mueller AM, Dohmann M, Spellerberg R, Urnauer S, Schwenk N, Ziegler SI, Nelson PJ & Spitzweg C, unpublished observations).

Thyroid hormone effects on mesenchymal stem cells

Data from our own laboratory has demonstrated that primary human bone marrow-derived mesenchymal stem cells treated with thyroid hormone in the presence of tumour cell-conditioned medium (human hepatocellular carcinoma) show enhanced expression of genes associated with a cancer-associated fibroblast-like phenotype (Fig. 1). These include the pro-angiogenic vascular growth factors VEGF, FGF2 and placental growth factor, in addition to HIF-1 α and pro-angiogenic angiogenin and angiopoietin 2. Furthermore, the expression of pericyte markers α -smooth muscle actin and desmin is stimulated, as well as the expression of less specific growth factors and cytokines that have been associated with angiogenesis, for example EGF, hepatocyte growth factor, insulin-like growth factor 1, TGF- β 1 and IL-6. These effects are all inhibited by tetrac (Schmohl *et al.* 2015; Schmohl KA, Mueller AM, Dohmann M, Spellerberg R, Urnauer S, Schwenk N, Ziegler SI, Nelson PJ & Spitzweg C, unpublished observations). Supernatant from thyroid hormone-stimulated mesenchymal stem cells enhances endothelial tube formation, while additional treatment with tetrac reduces tube formation to basal levels (Fig. 1) (Schmohl KA, Mueller AM, Dohmann M, Spellerberg R, Urnauer S, Schwenk N, Ziegler SI, Nelson PJ & Spitzweg C, unpublished observations). We further observed that both VEGF signalling and the hypoxia

Figure 1

Tetrac blocks the pro-angiogenic effects of thyroid hormones on mesenchymal stem cells. Mesenchymal stem cells, important progenitor cells of a tumour's fibrovascular network, play diverse roles during angiogenesis. As they express integrin $\alpha v \beta 3$, they are susceptible to thyroid hormone and tetrac stimulation. Thyroid hormones increase the differentiation of mesenchymal stem cells towards a cancer-associated fibroblast/pericyte-like phenotype in the tumour environment, which leads to secretion of pro-angiogenic modulators that in turn stimulate endothelial cell tube formation. They further stimulate pro-angiogenic signalling through activation of the VEGF promoter and a hypoxia-inducible promoter. In addition, recruitment of mesenchymal stem cells to tumours is enhanced under thyroid hormone stimulation. All these effects are blocked by additional treatment with tetrac. MSC: mesenchymal stem cell; CAF: cancer-associated fibroblast; ECs: endothelial cells; VEGF: vascular endothelial growth factor; HRE: hypoxia-responsive elements.

response in mesenchymal stem cells are subject to thyroid hormone activation via integrin $\alpha v \beta 3$ *in vitro* (Fig. 1). By applying MSCs with a VEGF promoter-driven reporter gene construct in an orthotopic hepatocellular carcinoma mouse model, we observed enhanced VEGF signalling in tumour-recruited MSCs in hyperthyroid mice as compared to euthyroid mice, while in hypothyroid or tetrac-treated animals, reporter gene expression was markedly reduced (Schmohl KA, Mueller AM, Dohmann M, Spellerberg R, Urnauer S, Schwenk N, Ziegler SI, Nelson PJ & Spitzweg C, unpublished observations).

In addition, thyroid hormone stimulates *in vitro* migration of mesenchymal stem cells towards tumour cell-conditioned medium and invasion into three-dimensional tumour cell spheroids, an effect that is again blocked by tetrac. Recruitment *in vivo* was analysed in a subcutaneous xenograft mouse model of human hepatocellular carcinoma. Mesenchymal stem cells showed significantly higher recruitment to the perivascular regions of tumours with strong infiltration into the surrounding tumour tissue in hyperthyroid mice as compared to euthyroid mice, while recruitment was significantly reduced in hypothyroid mice. In euthyroid mice treated with tetrac, recruitment was nearly completely abolished (Schmohl *et al.* 2015). The significance of this observation is that tetrac not only counteracts the differentiation of mesenchymal stem cells towards a more pro-angiogenic phenotype under

thyroid hormone stimulation, but also hampers their recruitment to their site of action in the tumour stroma (Fig. 1). Thus, mesenchymal stem cells as important tumour-supportive stroma cells have emerged as thyroid hormone-dependent targets via integrin $\alpha v \beta 3$.

Discussion

Only in recent years has it become clear that thyroid hormones act as small molecule pro-angiogenic modulators via integrin $\alpha v \beta 3$, and can support cancer-related angiogenesis as well as drive tumour growth (reviewed in Goemann *et al.* 2017). These effects are obviously undesirable in cancer patients, and endogenous as well as exogenously applied thyroid hormone may limit the effectiveness of anticancer therapy (Mousa *et al.* 2014, Lin *et al.* 2015). Indeed, clinical data and population-based studies have in part supported this notion.

Against this background, Herbergs *et al.* have proposed to reconsider T4 supplementation as standard hormone replacement therapy for hypothyroidism, and have raised the issue whether and how cancer patients in need of supplementation should be treated (Herbergs *et al.* 2016). Of note, in differentiated thyroid cancer patients, endogenous pituitary secretion of TSH, which stimulates differentiated thyroid cancer cell proliferation, is routinely suppressed by T4 administration. In patients with recurrence in spite

of full TSH suppression, the tumour may switch from TSH-dependency to dependency on exogenously administered T4 (Hercbergs *et al.* 2016). As detailed above, the affinity for T4 is far higher than for T3 at the integrin, which is consistent with an often reported higher responsiveness to T4 than to T3 in experimental studies, suggesting that T3 supplementation may have an advantage over T4 supplementation, at least in a subset of patients (Davis *et al.* 2013b, 2018, Hercbergs *et al.* 2015, 2016). Clearly, before these novel findings will be able to enter the clinical arena, more clinical data in larger patient cohorts are needed to elucidate the diverse effects of thyroid hormones and its derivatives on different aspects of cancer biology, such as proliferation, angiogenesis, inflammation, migration, as well as immunological aspects – especially considering the high variability seen in clinical studies that have been undertaken to date.

Increasing evidence suggests that most cancer-relevant thyroid hormone effects are mediated by integrin $\alpha\beta 3$. Therefore, blocking the integrin without affecting physiologic thyroid hormone signalling at nuclear TRs may be the more elegant, and less side effect-afflicted alternative – especially as the integrin is rarely expressed in high levels on normal cells. The advantage of tetrac over other integrin antagonists, specifically monoclonal antibodies or RGD-containing ligands, is that it does not *inactivate* the integrin, but allows selective manipulation of integrin function towards anticancer signalling (Davis *et al.* 2014b). Though focussed on a single specific receptor site, the anti-tumour actions of tetrac encompass multiple mechanisms downstream of the integrin. Besides interfering with numerous angiogenesis pathways, tetrac inhibits cancer cell proliferation, enhances cancer cell apoptosis and radio- and chemosensitises cancer cells (Rebbaa *et al.* 2008, Hercbergs *et al.* 2009, 2011, Lin *et al.* 2011, Davis *et al.* 2018). Furthermore, tetrac not only targets tumour endothelium and cancer cells themselves, but also affects mesenchymal stem cells as part of the tumour stroma. The tumour stroma plays a key role in tumour growth, angiogenesis and metastatic potential and has therefore emerged as a crucial target for cancer therapy. Tetrac blocks thyroid hormone-induced stimulation of differentiation of mesenchymal stem cells towards a tumour-promoting cancer-associated fibroblast-like phenotype, blocks angiogenic signalling and also significantly reduces recruitment and invasion of these cells into the tumour (Schmohl *et al.* 2015; Schmohl KA, Mueller AM, Dohmann M, Spellerberg R, Urnauer S, Schwenk N, Ziegler SI, Nelson PJ & Spitzweg C, unpublished observations).

Conclusions

In conclusion, over the last two decades, tremendous advances have been made in understanding the anti-angiogenic and general anticancer properties of tetrac. Tetrac possesses multiple favourable characteristics for clinical application, including high cell/tissue selectivity, low toxicity and low cost. Despite the cornucopia of promising preclinical data and a number of patents filed, (Nano)tetrac has not yet undergone clinical trials. Clinical evaluation of tetrac is an exciting prospect that will hopefully become a reality over the next decade.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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