UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE MEDICINA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS MÉDICAS: ENDOCRINOLOGIA

AVALIAÇÃO DOS EFEITOS DOS INIBIDORES TIROSINO-QUINASE NO METABOLISMO DOS HORMÔNIOS TIREOIDIANOS

CARLA DAIANA DEMKIO VOLASKO KRAUSE

Porto Alegre, Junho de 2017

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Orientadora: Profa. Dra. Ana Luiza Maia

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Esta dissertação de Mestrado segue o formato proposto pelo Programa de Pós-Graduação em Ciências Médicas: Endocrinologia, Metabolismo e Nutrição, Faculdade de Medicina, Universidade Federal do Rio Grande do Sul, sendo apresentada em duas partes:

- Parte I - Introdução e revisão da literatura;

- Parte II - Artigo original referente ao trabalho de pesquisa: Effect of Vandetanib on the Expression of Type 3 Deiodinase in Medullary Thyroid Carcinoma Cells.

LISTA DE ABREVIATURAS E SIGLAS

ATP - Adenosina trifosfato
CDT - Carcinoma diferenciado de tireoide
CFT - Carcinoma folicular de tireoide
c-Kit – Stem cell-factor receptor
c-MET – Hepatocyte growth factor
CMT - Carcinoma medular de tireoide
CPT - Carcinoma papilar de tireoide
D1/*DIO1* – Iodotironina desiodase tipo 1
D2/*DIO2* - Iodotironina desiodase tipo 2
D3/*DIO3* - Iodotironina desiodase tipo 3
DMSO - Dimethylsulfoxide
EGFR – Epidermal growth fator receptor
ERK – Extracellular-signal-regulated kinases
FLT-3 - FMS-like tyrosine kinase 3
ITQ/ITK – Inibidor tirosino-quinase
MAPK – Mitogen-activated protein kinase
mRNA – RNA mensageiro
NEM2 – Neoplasia endócrina múltipla tipo 2
PDGFR - Platelet-derived growth factor receptor
PLD - Progressão livre de doença
RET – RE-arranged during Transfection
rT3 – T3 reverso
SHH – Sonic hedgehog
T3 - Triiodotironina
T4 - Tiroxina
TGFR – Tumor growth factor receptor
TGFβ – Transforming growth factor beta
TSH - Hormônio estimulante da tireoide
VEGFR - Vascular endothelial growth factor receptor

SUMÁRIO

PARTE I

INTRODUÇÃO E REVISÃO DA LITERATURA

RESUMO

Introdução: Os inibidores tirosino-quinase (ITQs) constituem uma nova terapia molecular para o carcinoma medular da tireoide (CMT). O vandetanibe, um ITQ que atua contra os receptores VEGFR, EGFR e RET, inibe a transformação e o crescimento do tumor no CMT. No entanto, os ITQs têm importantes efeitos adversos, incluindo o hipotireoidismo. O aumento da expressão da iodotironina desiodase do tipo 3 (D3/*DIO3*), uma enzima chave na inativação dos hormônios da tireoide, pode ser um possível mecanismo de indução do hipotireoidismo por estas drogas.

Objetivo: Investigar os efeitos dos inibidores tirosino-quinase na expressão da D3 em células derivadas do CMT.

Métodos: Estudo experimental *in vitro*, utilizando linhagem de células humanas oriundas de CMT (células TT). As células foram cultivadas em meio específico e tratadas com diferentes doses do ITQ vandetanibe (0,25; 0,5 e 1µM) ou com DMSO. A proliferação celular foi determinada por contagem em câmara de Neubauer. A expressão do mRNA foi avaliada por meio de PCR em tempo real, a expressão proteica por meio de Western Blot e a atividade da D3 foi avaliada por meio da técnica de cromatografia em colunas de Sephadex LH-20.

Resultados: A adição do vandetanibe ao meio de cultura causou diminuição do número de células e seu efeito foi tempo e dose dependente, apresentando uma redução máxima (77%) após 6 dias de tratamento na dose de 1µM. Como esperado, o tratamento com vandetanibe inibiu a fosforilação do ERK. Não foram observadas alterações significativas dos níveis de mRNA da *DIO3* após 3 (0,02 vs. 0,02 vs. 0,01 vs. 0,01; *P* = 0,34) ou 6 dias (0,02 vs. 0,02 vs. 0,03 vs. 0,02; *P* = 0,33) de tratamento. Consequentemente, a expressão proteica da D3 não aumentou nos grupos tratados. No entanto, observou-se um aumento de 2 a 5 vezes na atividade da D3 após 3 dias de tratamento e um aumento de 1,5 a 2,15 vezes em 6 dias de tratamento.

Conclusões: O tratamento com vandetanibe não foi associado com níveis aumentados de expressão do mRNA e da proteína da D3 em células derivadas de CMT, embora tenha sido observado um aumento na sua atividade enzimática.

Palavras-chave: inibidores tirosino-quinase, câncer medular de tireoide, hipotireoidismo, desiodase tipo 3.

ABSTRACT

Background: Tyrosine kinase inhibitors (TKIs) constitute a novel molecular therapy for medullary thyroid carcinoma (MTC). Vandetanib, a TKI that acts against the VEGFR, EGFR and RET receptors, inhibits tumor transformation and growth in MTC. However, TKIs have important adverse effects, including hypothyroidism. Increases in the expression of type 3 iodothyronine deiodinase (D3/*DIO3*), a key enzyme in the inactivation of thyroid hormones, may be a possible mechanism of induction of hypothyroidism by these drugs.

Objective: To investigate the effects of vandetanib on D3 expression in MTC-derived cells.

Methods: *In vitro* experimental study using human MTC cell line (TT cells). Cells were cultured in specific medium and treated with different doses of vandetanib (0.25, 0.5 and 1µM) or DMSO. Cell proliferation was determined by counting in Neubauer's chamber. Expression of mRNA was evaluated by real-time PCR, protein expression by Western Blot and D3 activity was evaluated by Sephadex LH-20 column chromatography.

Results: The addition of vandetanib to the culture medium caused a time and dose-dependent decrease in the number of cells, with a maximum reduction (77%) after 6 days of treatment at 1µM dose. As expected, vandetanib treatment inhibited ERK phosphorylation. No significant changes in *DIO3* mRNA levels were observed after 3 (0.02 vs. 0.02 vs. 0.01 vs. 0.01; $P = 0.34$) or 6 days (0.02 vs. 0.02 vs. 0.03 vs. 0.02; $P = 0.33$) of treatment. Accordingly, D3 protein expression did not increase in treated groups. However, we observed a 2 to 5-fold increase in D3 activity after 3 days of treatment and a 1.5 to 2.15-fold increase in 6 days of treatment.

Conclusions: Treatment with vandetanib was not associated with increased *DIO3* mRNA and D3 protein expression levels in MTC-derived cells, although an increase in enzyme activity has been observed.

Keywords: tyrosine kinase inhibitors, medullary thyroid cancer, hypothyroidism, type 3 iodothyronine deiodinase.

INTRODUÇÃO

O câncer de tireoide constitui o tipo de neoplasia maligna mais comum do sistema endócrino, representando aproximadamente 1% de todas as neoplasias malignas humanas [1]. O carcinoma diferenciado de tireoide (CDT), originário da célula folicular tireoidiana, representa mais de 90% dos tumores da glândula e compreende o carcinoma papilar de tireoide (CPT, ~80% dos casos) e o carcinoma folicular de tireoide (CFT; ~10% dos casos). O carcinoma anaplásico de tireoide representa cerca de 1% dos tumores tireoidianos, podendo se originar *de novo* ou ser resultado da progressão e/ou desdiferenciação dos CDT.

O carcinoma medular de tireoide (CMT) é originado das células C ou parafoliculares tireoidianas e representa de 1-2% dos tumores tireoidianos [2]. O CMT pode ocorrer na forma hereditária ou esporádica (25 e 75% dos casos, respectivamente) [3, 4]. A forma hereditária apresenta-se como parte da síndrome de neoplasia endócrina múltipla tipo 2 (NEM 2), subdividida como NEM 2A (95% dos casos de NEM 2) e NEM 2B (5% dos casos de NEM 2). A primeira é caracterizada por CMT, feocromocitoma e hiperparatireoidismo, sendo mais frequentemente associada a mutações nos exons 10 e 11 do gene *RET.* A segunda é caracterizada por CMT, feocromocitoma, ganglioneuromatose e habitus marfanóides. NEM 2B é causada pela mutação M918T em aproximadamente 95% dos casos. Nesse subtipo, o CMT se desenvolve mais cedo e é mais agressivo que o do subtipo NEM 2A.

A forma esporádica é caracterizada pela presença de um nódulo palpável na tireoide ou por linfonodo cervical. O diagnóstico tende a ser mais tardio, entre a 5ª ou 6ª década de vida. Aproximadamente 50% dos pacientes desenvolvem metástase de linfonodo, enquanto que as metástases à distância ocorrem em cerca de 20% dos casos [5].

O tratamento do CMT é essencialmente cirúrgico. As opções terapêuticas para metástases à distância, como quimioterapia e radioterapia, são ineficientes e têm uma resposta limitada [6, 7], tornando o tratamento de pacientes com doença avançada e progressiva um desafio.

INIBIDORES TIROSINO-QUINASE

O conhecimento acumulado nos últimos anos sobre as distintas vias de sinalização e múltiplas anormalidades genéticas envolvidas na patogênese do câncer de tireóide possibilitou o desenvolvimento de terapias moleculares mais específicas, dentre elas os inibidores tirosino-quinase [5]. Estas drogas são pequenas moléculas que competem com a adenosina trifosfato (ATP), inibindo a autofosforilação e a transdução através de diferentes vias de sinalização [8]. Dentre elas, as quinazolinas se enquadram entre os mais promissores inibidores de receptores tirosino-quinase ou de suas vias de comunicação. Atuam inibindo a atividade quinase do receptor do fator de crescimento epidérmico (EGFR), do fator de crescimento do endotélio vascular (VEGFR), do receptor RET-TK, do fator de crescimento tumoral (TGFRα), do fator de crescimento do hepatócito (HGFR), entre outros [9]. A seguir, descrevemos sumariamente os principais inibidores tirosino-quinases utilizados no tratamento de neoplasias.

O sunitinibe é um inibidor de receptor tirosino-quinase que tem potente atividade anti-angiogênica e antitumoral, atuando através da inibição dos receptores RET, VEGFR-1, VEGFR-2, PDGFR-α e PDGFR-β, c-Kit e FLT-3 [10, 11]. Atualmente, o maleato de sunitinibe está indicado para o tratamento de tumor estromal gastrintestinal e de carcinoma metastático avançado de células renais. No entanto, baseado no mecanismo de ação desta medicação, é possível que atue também nos casos de carcinoma medular de tireoide. Em concordância com esta hipótese, há na literatura a descrição de casos de pacientes com carcinoma medular de tireoide metastático que apresentaram resposta satisfatória ao tratamento com sunitinibe na dose de 50 mg por dia [12, 13].

O sorafenibe é também um eficaz inibidor da atividade de tirosino-quinase do RET, VEGFR-2, VEGFR-3, PDGFR-β, c-Kit e FLT-3 [14]. Estudos demonstraram uma forte inibição no crescimento de células tumorais com rearranjo RET-PTC1 injetadas em camundongos [15]. Outro estudo revelou uma importante redução no crescimento das células TT e células NIH3T3 transfectadas com oncogene *RET*. Após 3 semanas de tratamento de ratos com implante das células TT, o volume tumoral reduziu significativamente em relação aos controles [16]. Em um pequeno estudo piloto, foi evidenciada uma resposta em 2 pacientes de 5 com CMT

metastático, após 6 meses de tratamento com sorafenibe, sendo que em um deles a resposta foi completa, além de uma melhora sintomática em todos os pacientes [17]. Em um ensaio clínico de fase II, pode-se observar uma resposta parcial de 6,3% em pacientes com CMT esporádico tratados com 400 mg/dia de sorafenibe e 87,5% tiveram estabilização da doença, com uma taxa de sobrevida livre de doença de 17,9 meses [18].

O cabozantinibe é um inibidor tirosino-quinase do receptor do fator de crescimento do hepatócito (MET), do VEGFR2 e do RET, e que também foi aprovado para o tratamento de CMT avançado. Ele se mostrou eficaz no tratamento de pacientes com carcinoma medular de tireoide em um estudo clínico de fase I [19]. Em outro estudo de fase III, o cabozantinibe demonstrou um aumento significativo na taxa de progressão livre de doença (PLD) em pacientes com CMT metastático (11,2 meses para o grupo tratado versus 4 meses para o grupo placebo) [20].

O vandetanibe (Fig. 1), uma anilonaquinazolina que inibe seletivamente os subtipos de receptor VEGF-2, EGFRs e RET tem sido empregado com sucesso em pacientes com diversos tipos de carcinomas [21-23]. Alguns estudos pré-clínicos com câncer de pulmão, pâncreas e estômago, evidenciaram que o vandetanibe inibe o crescimento tumoral, a formação de novos vasos e a permeabilidade vascular tumoral [23, 24]. Além disso, os dados demonstram que o vandetanibe possui um potencial efeito inibitório de metástases, prevenindo a disseminação do tumor primário [25, 26]. Estudos *in vitro* evidenciaram que o vandetanibe também pode inibir diretamente o crescimento de linhagens tumorais de pulmão, ovário, intestino e mama, através da inibição da atividade do EGFR, uma via de sinalização central na progressão tumoral. A magnitude dessa inibição parece depender da expressão tumoral de EGFR mutante [21]. Carlomagno *et al* (2002), em experimentos feitos em cultura de células e em tumores enxertados em ratos, demonstraram que o vandetanibe inibe a fosforilação e a sinalização do oncogene RET/PTC nos casos de CPT associados a esse rearranjo, impedindo o crescimento tumoral e, inibindo o VEGF-2, bloqueando as oncoproteínas derivadas do RET [27].

Estudos mais recentes avaliando a resposta ao vandetanibe em pacientes com CMT evidenciaram taxas de resposta parcial entre 16-20% e de estabilização da doença (por 24 semanas ou mais) de 53% [28, 29]. O vandetanibe foi avaliado para o CMT avançado em um grande estudo clínico randomizado, onde foi observada uma melhora significativa na sobrevida livre de progressão da doença

nos pacientes tratados com o inibidor, em comparação ao grupo que recebeu placebo [29]. Em um estudo clínico de fase I/II, o vandetanibe também mostrou ser eficaz para o tratamento de crianças e adolescentes com CMT [30].

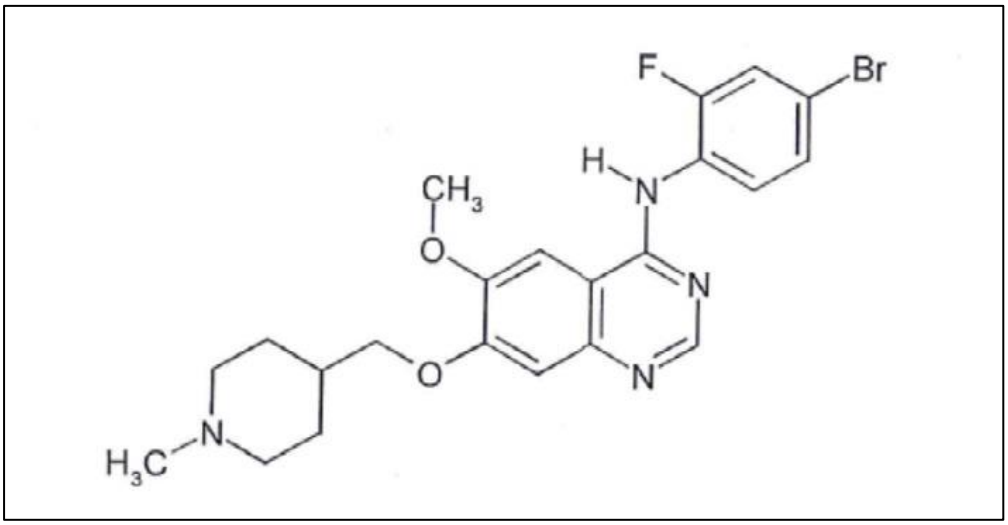

Figura 1: Estrutura química do inibidor tirosino-quinase vandetanibe.

Outras drogas inibidoras de receptores tirosino-quinase como o imatinibe, motesanibe e axitinibe têm sido amplamente estudadas para o tratamento dos carcinomas tireoidianos [5]. A tabela 1 apresenta o resultado dos principais estudos de tratamento do CMT com inibidores tirosino-quinase.

Dentre os efeitos adversos destas drogas como diarreia, náuseas, erupções cutâneas, fadiga e dor de cabeça [31], o hipotireoidismo (evidenciado por elevados níveis de TSH) destaca-se como um dos mais importantes [32]. Têm sido relatados ajustes no tratamento hormonal tireoidiano de substituição em pacientes tireoidectomizados sob terapia com sunitinibe ou imatinibe, com a necessidade de até 350% de aumento na dose de levotiroxina [33]. No entanto, os mecanismos que levam a esta disfunção da tireoide não estão elucidados.

Tabela 1: Resultados dos estudos com inibidores tirosino-quinase em CMT.

Drogas investigadas	**Alvos moleculares**	**Resposta parcial/estabilização da doença (%)**	**Referências**
Motesanibe	VEGFR 1-3, c-KIT, RET, PDGFR	2/48	[34]
Sorafenibe	VEGFR 2-3, c-KIT, RET	6/50	[18], [35]
Sunitinibe	VEGFR 1-3, c-KIT, RET	28/46	[36]
Axitinibe	VEGFR 1-3, c-KIT	18/27	[37]
Imatinibe	c-KIT, RET, PDGFR	0/27	[38]
Lenvatinibe	VEGFR-1–3, FGFRs1–4,PDGFRα, RET, c-KIT, SCFR	50/43	[39]
Drogas aprovadas	**Alvos moleculares**	**PLD droga vs. Placebo (meses)**	**Referências**
Vandetanibe	VEGFR 1-3, RET, EGFR	30,5 vs 19,3	[31]
Cabozantinibe	VEGFR 2, RET, c-MET	11,2 vs 4,0	[20]

PLD: progressão livre da doença. Adaptado de Maia *et al*, 2017 [40].

HIPOTIREOIDISMO INDUZIDO PELOS INIBIDORES TIROSINO-QUINASE

O hipotireoidismo primário refere-se a uma diminuição da produção do hormônio da tireoide, o que provoca um aumento nos níveis de TSH, sendo a tireoidite autoimune crônica a causa mais frequente. O hipotireoidismo central tem múltiplas causas: tumores, traumas, infecções vasculares, infiltrativas, inflamatórias ou congênitas. Além da perda de tecido funcional, o hipotireoidismo central também pode resultar de defeitos funcionais na biossíntese ou liberação do TSH devido a mutações genéticas ou a drogas, como a dopamina e glicocorticoides [41]. Os sintomas característicos do hipotireoidismo incluem fadiga, ganho de peso, intolerância ao frio, entre outros [42].

Em um estudo publicado em 2008, Dora e colaboradores mostraram que pacientes não-tireoidectomizados sob tratamento com imatinib não desenvolveram

hipotireoidismo [43], afastando os hipotireoidismos primário e central como causa da deficiência dos hormônios tireoidianos induzida pela terapia com essas drogas. O aumento da demanda por levotiroxina induzida por inibidores tirosino-quinase em pacientes em reposição do hormônio tireoidiano [33] pode indicar uma alteração no metabolismo periférico dos hormônios da tireoide [43].

METABOLISMO PERIFÉRICO DOS HORMÔNIOS TIREOIDIANOS

Os hormônios tireoidianos são responsáveis pela regulação do crescimento, desenvolvimento e gasto energético em humanos, além de exercerem papel fundamental no equilíbrio entre proliferação e diferenciação celular em todos os órgãos [44]. As iodotironinas desiodases tipos 1, 2 e 3 (D1/*DIO1*, D2/*DIO2* e D3/*DIO3*) constituem uma família de oxiredutases que catalizam a remoção de uma molécula de iodo do anel externo (D1 e D2, ativação) e do anel interno (D3, inativação) dos hormônios tireoidianos [45]. Estas enzimas contêm o raro aminoácido selenocisteína em seu sítio ativo, um resíduo essencial para uma atividade catalítica eficiente [46-48] e foram identificadas como membros da superfamília das tioredoxinas (TRX)-fold [49].

A via da desiodação é um passo crítico na ativação e inativação do hormônio da tireoide, permitindo rápidas modificações no status tireoidiano intracelular de uma forma tecido-específica, sem afetar as concentrações circulantes dos mesmos. Assim, é possível controlar a concentração e a atividade intracelular de T3 independentemente dos níveis de T3 sérico. A monodesiodação do T4 a T3 ocorre via ação das enzimas D1 e D2, que respondem por cerca de 80 % da produção diária de T3 em humanos (Fig. 2). A D2 é uma desiodase exclusiva por atuar no anel externo que converte T4 em T3 e rT3 (T3 reverso) em T2. Ao contrário, a D1 pode promover a desiodação tanto do anel externo quanto do anel interno (Fig. 2). Em humanos, os níveis mais altos de atividade da D1 são encontrados na tireoide, fígado e rim. A D2 é mais expressa na hipófise, cérebro, tireoide, pele, músculos esquelético e cardíaco [45, 50]. A D3 desempenha exclusivamente atividade de desiodação do anel interno, catalisando a conversão de T4 a rT3 e a conversão de T3 a 3,3'-T2, ambos produtos biologicamente inativos (Fig. 2).

A D3 contribui para a homeostase do hormônio da tireoide, protegendo os

tecidos de um excesso de hormônio tireoidiano. Durante a embriogênese, a D3 é essencial para o programa de desenvolvimento fetal, pois a exposição excessiva ou prematura de embriões humanos ao T3 pode resultar em malformações, alterações no crescimento, retardo mental ou até mesmo morte. Como demonstrado em vários modelos animais fetais e neonatais, a expressão de D3 é altamente regulada seguindo um padrão tecido-específico, o qual é provavelmente essencial para a regulação coordenada dos efeitos dos hormônios tireoidianos no desenvolvimento. A D3 também desempenha um papel fundamental na fisiopatologia da síndrome do T3 baixo em humanos [51].

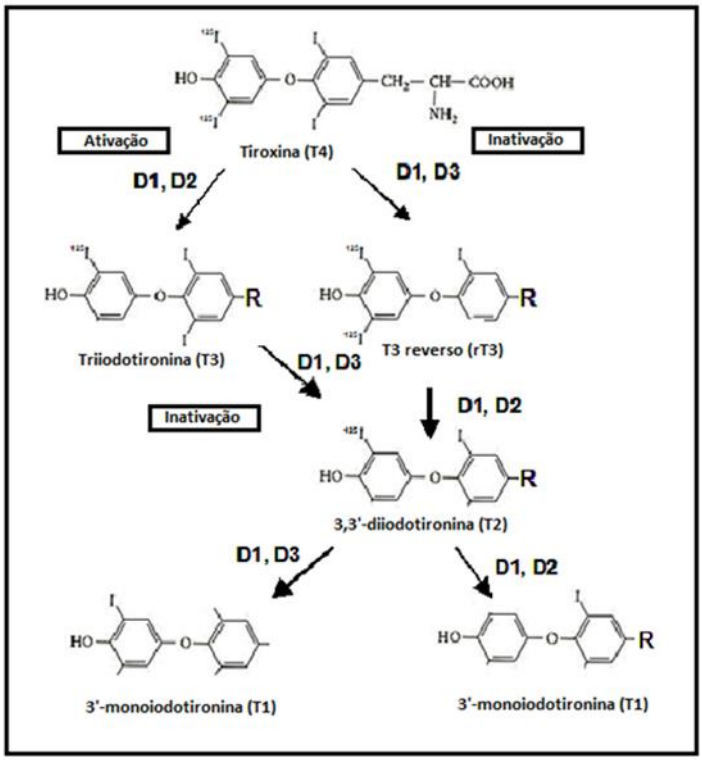

Figura 2: Processo de desiodação dos hormônios tireoidianos promovido pelas enzimas iodotironinas desiodases tipos 1, 2 e 3 (D1, D2 e D3).

METABOLISMO PERIFÉRICO VS. INIBIDORES TIROSINO-QUINASE

Alterações nas desiodases têm sido sugeridas como potencial mecanismo para o hipotireoidismo induzido pelos inibidores tirosino-quinase. Em um estudo

recente em ratos tratados com sunitinibe, foi relatada uma diminuição da concentração sérica de T4 e T3, acompanhada por um aumento da atividade da D3 hepática e atividade reduzida da D1 e uma histologia da tireoide mostrando regressão capilar [32]. Abdulrahman e colaboradores, em um estudo com pacientes tireoidectomizados tratados com sorafenibe, verificaram redução nos níveis séricos de T3 e T4 livres (provenientes da administração de levotiroxina), com aumento nos níveis de TSH e diminuição do T3/T4 e T3/rT3, sugerindo provável aumento da atividade da D3 [52].

JUSTIFICATIVA

Os inibidores tirosino-quinase são uma nova e promissora classe de terapia contra o câncer metastático. Estudos recentes mostraram que um dos efeitos adversos mais importantes destas drogas é o hipotireoidismo, evidenciado pelos elevados níveis de TSH e necessidade de aumento significativo da dose de hormônio de substituição em pacientes tireoidectomizados. As iodotironinas desiodases são importantes reguladores da biodisponibilidade dos hormônios da tireoide. No entanto, não está elucidado o efeito dos inibidores tirosino-quinase sobre essas moléculas, que podem estar envolvidas nos mecanismos que levam à desregulação dos hormônios tireoidianos causada pelo tratamento com estas drogas.

OBJETIVO

Investigar o efeito do inibidor tirosino-quinase vandetanibe na expressão e atividade enzimática da D3 em células derivadas de CMT.

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PARTE II

EFFECT OF VANDETANIB ON THE EXPRESSION OF TYPE 3 DEIODINASE IN MEDULLARY THYROID CARCINOMA CELLS

Effect of Vandetanib on the Expression of Type 3 Deiodinase in Medullary Thyroid Carcinoma Cells

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Running title: Vandetanib and D3 expression in MTC cells

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Abstract

Background: Tyrosine kinase inhibitors (TKIs) constitute a novel molecular therapy for medullary thyroid carcinoma (MTC). Vandetanib, a TKI that acts against the VEGFR, EGFR and RET receptors, inhibits tumor transformation and growth in MTC. However, TKIs have important adverse effects, including hypothyroidism. Increases in the expression of type 3 iodothyronine deiodinase (D3/*DIO3*), a key enzyme in the inactivation of thyroid hormones, may be a possible mechanism of induction of hypothyroidism by these drugs.

Objective: To investigate the effects of vandetanib on D3 expression in MTC-derived cells.

Methods: *In vitro* experimental study using human MTC cell line (TT cells). Cells were cultured in specific medium and treated with different doses of vandetanib (0.25, 0.5 and 1μM) or DMSO. Cell proliferation was determined by counting in Neubauer's chamber. Expression of mRNA was evaluated by real-time PCR, protein expression by Western Blot and D3 activity was evaluated by Sephadex LH-20 column chromatography.

Results: The addition of vandetanib to the culture medium caused a time and dose-dependent decrease in the number of cells, with a maximum reduction (77%) after 6 days of treatment at the 1μM dose. As expected, vandetanib treatment inhibited ERK phosphorylation. No significant changes in *DIO3* mRNA levels were observed after 3 (0.02 vs. 0.02 vs. 0.01 vs. 0.01; $P = 0.34$) or 6 days (0.02 vs. 0.02 vs. 0.03 vs. 0.02; $P = 0.33$) of treatment. Accordingly, D3 protein expression did not increase in treated groups. However, we observed a 2 to 5-fold increase in D3 activity after 3 days of treatment and a 1.5 to 2.15-fold increase in 6 days of treatment.

Conclusions: Treatment with vandetanib was not associated with increased *DIO3* mRNA and D3 protein expression levels in MTC-derived cells, although an increase in enzyme activity has been observed.

Keywords: tyrosine kinase inhibitors, medullary thyroid cancer, hypothyroidism, type 3 iodothyronine deiodinase.

Introduction

Thyroid cancer accounts for approximately 1% of all cancers [1] and is the most common malignant endocrine tumor. Medullary thyroid carcinoma (MTC) originates from C-cells or thyroid parafollicular cells and accounts for 1-2% of thyroid tumors [2]. MTC may occur in hereditary (25% of cases) or sporadic (75% of cases) forms [3, 4]. Hereditary MTC presents as part of the syndrome of multiple endocrine neoplasia type 2 (MEN 2), sub-divided as MEN 2A (95% of MEN2 cases) and MEN 2B (5% of MEN2 cases) [2].

The treatment of the MTC is essentially surgical. Therapeutic options for distant metastases such as chemotherapy and radiotherapy are ineffective and have a limited response [5-7], making treatment of patients with advanced and progressive disease a challenge. However, the cumulative knowledge of the different signaling pathways and multiple genetic abnormalities involved in the pathogenesis of thyroid cancer has allowed the development of target molecular therapies, among them tyrosine kinase inhibitors (TKIs). The protein kinases regulate the processes of cell proliferation, differentiation, migration and anti-apoptosis signaling. These proteins are characterized by their ability to catalyze the phosphorylation of amino acid residues tyrosine in proteins and thus activate several cascades of intracellular signaling. TKIs bind to receptors and compete with adenosine triphosphate (ATP) at catalytic binding site of tyrosine kinase. Thus, TKIs can act as a therapy for cancer by blocking tyrosine kinase-dependent oncogenic pathways.

Vandetanib is a TKI that acts against the VEGFR (vascular endothelial growth factor receptor), EGFR (epidermal growth factor receptor) and RET (Rearranged during Transfection) receptors. In human cell lines derived from MTC, vandetanib inhibited cell proliferation and phosphorylation of RET receptor, EGFR and MAPK (Mitogen Activated Protein Kinase) pathway [8]. Vandetanib was evaluated for advanced MTC in a large randomized clinical trial, where a significant improvement in disease progression-free survival was observed in patients treated with the inhibitor as compared to the placebo group [9].

Hypothyroidism, evidenced by high levels of TSH, stands out as one of the most frequent adverse events reported by the use of TKIs [10]. Adjustments in thyroid hormone replacement therapy have been reported in thyroidectomized patients under sunitinib or imatinib therapy, with a need for up to 350% increase in

levothyroxine dose [11]. Studies with vandetanib in patients with MTC have also shown a need to increase the dose of levothyroxine [9, 12, 13].

The exact mechanisms that lead to thyroid dysfunction are not yet known. The increased demand for levothyroxine induced by TKIs in patients undergoing thyroid hormone replacement may indicate a change in the peripheral metabolism of thyroid hormones [11, 14]. Thyroid hormone metabolism is coordinated by the enzymes iodothyronine deiodinases types 1, 2 and 3 (D1 / *DIO1*, D2 / *DIO2* and D3 / *DIO3*), which are responsible for the activation and inactivation of thyroid hormone (TH). D3 is the main inactivator of TH, and recent studies suggest a relationship between increased activity of this enzyme and the development of hypothyroidism during treatment with TKIs. Abdulrahman and colleagues, in a study with thyroidectomized patients treated with sorafenib, found a reduction in free T3 and T4 serum levels (from levothyroxine administration), with an increase in TSH levels and a decrease in T3 / T4 and T3 / rT3, suggesting an increase in D3 activity [15]. In a study using rats, sunitinib induced increased liver D3 activity [16]. Maynard *et al* (2014) described severe hypothyroidism in a GIST (gastrointestinal stromal tumor) patient prior to starting treatment with sunitinib and showed that this was caused by overexpression and D3 activity in the tumor. In the same study, sorafenib and sunitinib were shown to increase D3 levels in a GIST-T1 cell line, which might contribute to the development of hypothyroidism during treatment with TKIs [17].

Considering that hypothyroidism is an important adverse effect of vandetanib and that, to date, no study has been conducted to investigate its effect on the D3 expression in MTC, we performed studies to investigate this possible mechanism of action which leads to this dysfunction.

Materials and Methods

Cell culture

TT cell line (no. CRL 1803, obtained from Banco de células do Rio de Janeiro) is derived from a human MTC and harbors a MEN 2A-type mutation, characterized by a cysteine to tryptophan substitution at the level of *RET* codon 634 [18]. Cells were cultured in RPMI-1640 (Life Technologies, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Invitrogen) and maintained at 37ºC in a humidified atmosphere of 5% CO_2 and 95% air. The culture

medium was changed three times a week.

Tyrosine kinase inhibitor treatment

Vandetanib (AstraZeneca, Zoetermeer, The Netherlands) stock solution was made in 100% dimethylsulfoxide (DMSO). Equivalent DMSO concentration (0.1%) served as control.

TT cells were treated with 0.25, 0.5 and 1 µM of vandetanib for different intervals of time. Cell culture medium was replenished every day. Cells were collected after 0 - 6 days and processed for cell proliferation assay, total RNA and protein extraction and for D3 activity assay. All experiments were performed in duplicate or triplicate in at least two independent experiments.

Cell proliferation assay

TT cells were plated at concentration of 5 x 10^5 cells/well in six-well plates. Increasing concentrations of vandetanib solution (0.25, 0.5 and 1 µM) were added. A concentration of 0.001% DMSO was used as vehicle. Control cells were grown without DMSO or vandetanib. Proliferation was measured at 2, 4 and 6 days and the absolute number was counted using the Neubauer's chamber.

RNA extraction and real-time PCR

Total RNA was extracted using the RNeasy minikit (Qiagen). One microgram of RNA was reverse transcribed into cDNA using the SuperScript VILO MasterMix (Life Technologies, Invitrogen), following the manufacturer's protocol. RT-PCR experiments were performed in a 7500 Fast Real-Time PCR System Thermal Cycler with 7500 FAST System Sequence Detection 1.4 Software (Life Technologies, Applied Biosystems). The following primers were used: human *DIO3* gene (forward primer 5´-TTCCAGAGCCAGCACATCCT-3´ and reverse primer 5´-ACGTCGCGCTGGTACTTAGTG-3') and cyclophilin A as reference gene (forward primer 5'-GTCAACCCCACCGTGTTCTTC-3' and reverse primer 5'-ACTTGCCACCAGTGCCATTATG-3'). Each sample was assayed in triplicate. Quantification of *DIO3* cDNA was performed by relative quantification using the

comparative ΔΔCT method and expressed relative to the reference gene (cyclophilin A). Changes in gene expression were expressed as arbitrary units.

Western blot analysis

TT cells were lysed in a RIPA solution and lysates were clarified by centrifugation at 14000 rpm for 15 minutes. Total protein was spectrophotometrically quantified with the Bradford method. Briefly, 30 µg protein of each sample was fractionated by 4–12% SDS–PAGE and blotted onto an Immobilon PVDF membrane (Millipore, Billerica, MA, USA). Non-specific binding sites were blocked by incubation with 5% BSA in Tris-buffered saline 0.1% Tween-20. The following primary antibodies were used: anti-D3 (1:500; Novus Biologicals, Littleton, CO, USA), anti-ERK1/2 (1:400; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phospho-ERK1/2 (1:400; Santa Cruz Biotechnology) and anti-β-actin (1:10000; Sigma–Aldrich). The antigen–antibody complexes were visualized using HRP-conjugated secondary antibody and an enhanced chemiluminescence system (GE Healthcare, Pittsburgh, PA, USA). Expression was quantified using image densitometry with ImageJ analysis software.

D3 activity assay

D3 activity was determined in TT cells by Sephadex LH-20 column chromatography [19]. After concluding the experiments, cells were harvested and sonicated with 10 mM Tris-HCl, 0.25 sucrose buffer (pH 7.5). Sonicated cells were incubated for 1 hour with 200000 cpm (^{125}I)T3 (American Radiolabeled Chemicals, Saint Louis, MO, USA), 2 nM T3 and 20 mM DTT. The reaction was stopped with 200 µl horse serum and 100 µl 50% TCA, which was followed by centrifugation at 12 000 g for 2 min to precipitate the non-metabolized (^{125}I)T3. The supernatant was used to determine (^{125}I)T2 and (^{125}I)T1 levels. The Sephadex LH-20 column (Sigma-Aldrich) was equilibrated with 0.1 M HCl, and an equal volume of 0.1 M HCl was added to 500 µl samples and then mixed. Stepwise elution was performed by successive application of 2 x 1 ml of 0.1 M HCl (for ^{125}I release), 4 x 1 ml of 20% ethanol (for [^{125}I] T1 release) and 2 x 1 ml of 50% ethanol in 0.1 MNaOH (for [^{125}I]T2 release). The 1-ml fractions were collected and counted for radioactivity. D3 activity was

calculated by multiplying the fractional conversion by the T3 concentration in the media and expressed as T3 inactivation (fmol/mg protein/min).

Statistical Analysis

DIO3 mRNA was expressed as arbitrary units, while D3 activity and the number of cells as mean ± S.D. To compare D3 levels among the groups, we used Student's t-test or one-way ANOVA. Statistical analyzes were performed by software GraphPad Prism, version 6.0. P <0.05 was considered statistically significant.

Results

Effect of vandetanib on cell proliferation

Initially, we evaluated the proliferation of TT cells treated with different concentrations of vandetanib or DMSO. A dose- and time-dependent decreased in cell proliferation was observed. Cell growth was not significantly affected by treatment with DMSO (Fig. 1). An antiproliferative effect can be observed from the second day of treatment at all doses, and this effect became stronger on the fourth day, in which we observed a significant reduction of approximately 62% in the number of cells at 1 μM dose (P=0.001). After 6 days of treatment, TT cells treated with 1μM vandetanib had a significantly lower count (162×10^3) than those treated with vehicle (702×10^3). This difference corresponds to 77% of cell number reduction (difference=540×10^3, 95% confidence interval CI=398-682×10^3, P<0.001) (Fig.1). Based on these results, we decided to evaluate the effect of vandetanib on cells treated for 3 and 6 days. After 6 days, cell proliferation drastically reduces and the vast majority of cells are no longer viable.

Effect of vandetanib on inhibition of ERK phosphorylation

We also analyzed the inhibitory effect of vandetanib on ERK phosphorylation, one of the elements of the MAPK pathway, which is activated by RET. On the third day, ERK phosphorylation was inhibited at the 0.25 and 0.5 μM doses, but not at the 1 μM dose. Vandetanib decreased ERK phosphorylation after 6 days of treatment, at

0.25 and 1 µM doses, but not at 0.5 µM (Fig. 2).

Effect of vandetanib on DIO3 mRNA and protein levels

To evaluate the effect of vandetanib on *DIO3* mRNA expression, we performed quantitative PCR. We did not observe significant variation in *DIO3* mRNA levels after 3 (0.02 vs. 0.02 vs. 0.01 vs. 0.01; *P* = 0.34) or 6 days (0.02 vs. 0.02 vs. 0.03 vs. 0.02 ; *P* = 0.33) of treatment (Fig.3).

We also investigated D3 protein expression levels by immunoblotting with specific antibody. On the third day, we observed a small decrease in protein levels at the 0.25 and 0.5 µM doses and a small increase at the 1µM dose. On the sixth day, there was no increase in expression levels at the 0.25 and 0.5 µM doses, but there was a decrease at the 1µM dose, compared to control (Fig. 4).

Effect of vandetanib on D3 activity

Next, we evaluated the effect of vandetanib on D3 activity. No D3 activity was detectable in control cells, whereas cells treated with different doses of vandetanib show a 2 to 5-fold increase in D3 activity after 3 days of treatment. Interestingly, contrasting with what was observed at 3 days, control cells exhibited D3 activity on the sixth day (4.29 pmol/mg prot/min). Similarly, we observed a 1.5 to 2.15-fold increase in activity in the treated groups (Fig. 5).

Discussion

Medullary thyroid carcinoma is a rare malignant tumor, accounting for 1-2% of thyroid neoplasias. Until recently, the treatment of patients with advanced or metastatic disease was a clinical challenge, since chemotherapy and radiotherapy do not present satisfactory results. Nevertheless, the development of new target molecular therapies, among them TKIs, has shown promising results. Vandetanib was the first approved TKI to treat advanced MTC and have been shown to improve progression-free survival in these patients [20]. Despite the satisfactory results for the treatment of MTC, TKIs still have important adverse effects, including

hypothyroidism. Patients receiving TKI treatment need to increase levothyroxine dose in thyroid hormone replacement therapy [11-13]. Increases in D3 expression may be a possible mechanism of induction of hypothyroidism by these drugs.

In this study, we have confirmed the antiproliferative effect of vandetanib on MTC-derived cells. Micromolar concentrations of the drug reduced cell proliferation in a dose and time-dependent manner, which is consistent with previous results showing a growth inhibitory effect of vandetanib on *RET* mutant MTC cells [8]. We also explored the effect of vandetanib on MAPK pathway, a downstream signaling pathway directly activated by RET. ERK is one of the elements of MAPK pathway and ERK phosphorylation influences cell proliferation, differentiation, transcription regulation and development. The drug induced a decrease in ERK phosphorylation, although this inhibition has been fluctuating (Fig. 2). These inhibitory effects may be overcome by the compensatory activation of other kinases [21]. Other receptor or TK effector molecules may exert a stronger effect on ERK phosphorylation than RET [22]. Inhibition of phosphorylation may also be correlated with decreased cell proliferation, as we can observe in figure 1.

Nevertheless, we failed to demonstrate vandetanib-related effect on *DIO3* mRNA and protein levels in MTC-derived cells. Vandetanib did not increase significantly *DIO3* mRNA or D3 protein levels after 3 or 6 days of treatment. *DIO3* gene is upregulated at transcriptional level and this upregulation might be driven by disruption of several signaling pathways such as MAPK, transforming growth factor beta (TGFβ), sonic hedgehog (SHH) and β-catenin [23]. Vandetanib inhibits MAPK pathway, resulting in decreased *DIO3* transcription, but this can be offset by the activation of other pathways such as SHH. Here, we showed vandetanib did not increase *DIO3* mRNA expression levels and this effect may be due to inhibition of MAPK pathway.

Our results contrast with a previous study conducted with another TKI (sorafenib), which suggests that the decreased serum thyroid hormone levels during the treatment is compatible with increased type 3 deiodination [15]. Maynard and colleagues investigated the effect of other TKIs (imatinib and sunitinib) on D3 expression in gastrointestinal stromal tumor cells (GIST-T1), neuroblastoma cells (SK-N-AS) and breast-cancer cells (MCF-7). Of interest, *DIO3* mRNA levels increased in GIST-T1 cells, but not in SK-N-AS or in MCF-7 cells, showing that the effect might be cell-type specific [17].

As far as we know, we were the first to investigate the effects of vandetanib on D3 expression in MTC-derived cells. Several reasons may explain our negative results in mRNA and protein expression. Although the doses used in this study were based on published data [8, 22], these may be too low to induce any significant increase in D3 expression. Additionally, the treatment time may be insufficient to result in any increase. We used TT cell line because it is representative of MTC. However, it is possible that this cell type, that does not express D3, is not responsible for D3 expression in patients who have the disease. Thus, it would be interesting to test the effect of vandetanib on other cell lines that naturally express D3.

Interestingly, we observed an increase in activity, contrasting with was observed in *DIO3* mRNA expression levels. It is possible that this protein is being regulated by post-translational mechanisms that allow the increase of half-life or its stability, such as de-ubiquitination. This mechanism is known for D2, but has not yet been described for D1 and D3 [24]. In addition, D3 is sensitive to redox alterations [19] and activity may increase when cells are exposed to oxidative stress environment. Vandetanib might be causing this condition, explaining why activity increased and mRNA levels did not. Our D3 activity results are in agreement with a study which evaluated the effect of sunitinib in rats showed that TKI treatment was associated with increased hepatic D3 activity. After sunitinib withdrawal, changes in D3 activity were reversible, demonstrating that the inhibitor actually induced increased expression of this protein [16]. Increased D3 activity by a factor of as much as 1.8 was also observed in GIST-T1 cells treated with TKI in the study by Maynard *et al* [17].

In conclusion, our results indicate that treatment with vandetanib was not associated with increased *DIO3* mRNA and protein expression levels in MTC-derived cells, although an increase in enzyme activity has been observed and might be explained by post-translational regulation mechanisms. Thus, further testing will be required to investigate this hypothesis.

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Figures

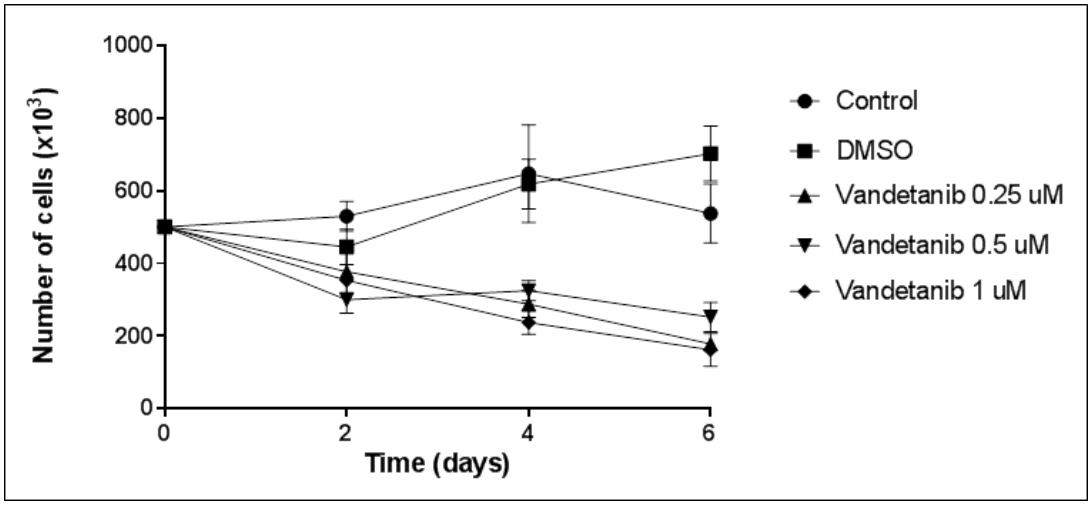

Figure 1: Effect of vandetanib on TT cell proliferation. Cells were incubated with vehicle (DMSO) or increasing concentrations of vandetanib (0.25, 0.5 and 1 µM) and counted at 2, 4 and 6 days. The maximal cell-count reduction was observed after 6 days of exposure to 1µM vandetanib (77% of cell number reduction, $P<0.001$).

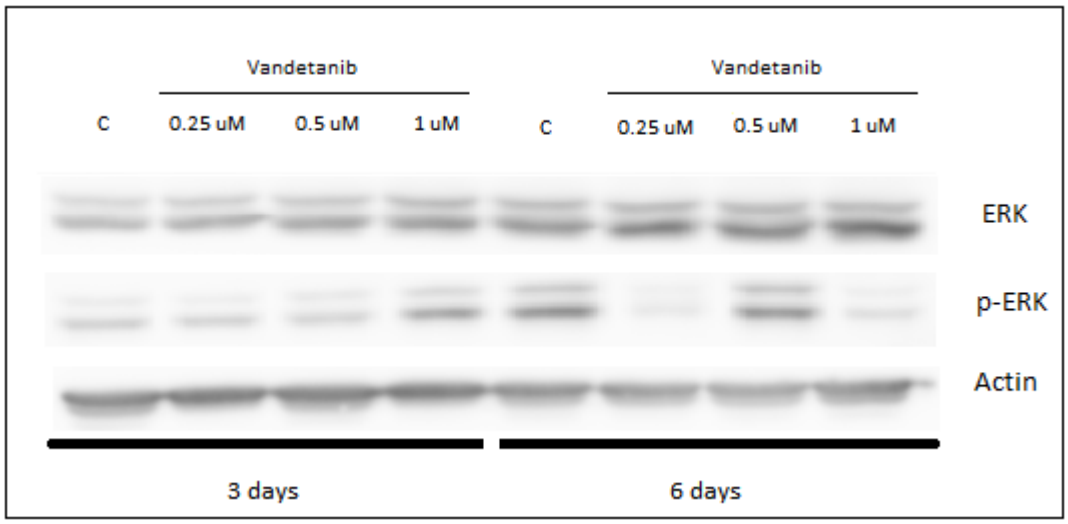

Figure 2: Effect of vandetanib on inhibition of ERK phosphorylation. Cells were treated with different concentrations of vandetanib; 30 µg of total cells lysates were immunoblotted with specific antibodies for ERK and phosphorylated ERK. Vandetanib inhibited ERK phosphorylation on the third day at 0.25 and 0.5 µM doses, but not at 1 µM dose. After 6 days of treatment, ERK phosphorylation was inhibited at 0.25 and 1 µM doses, but not at 0.5 µM dose.

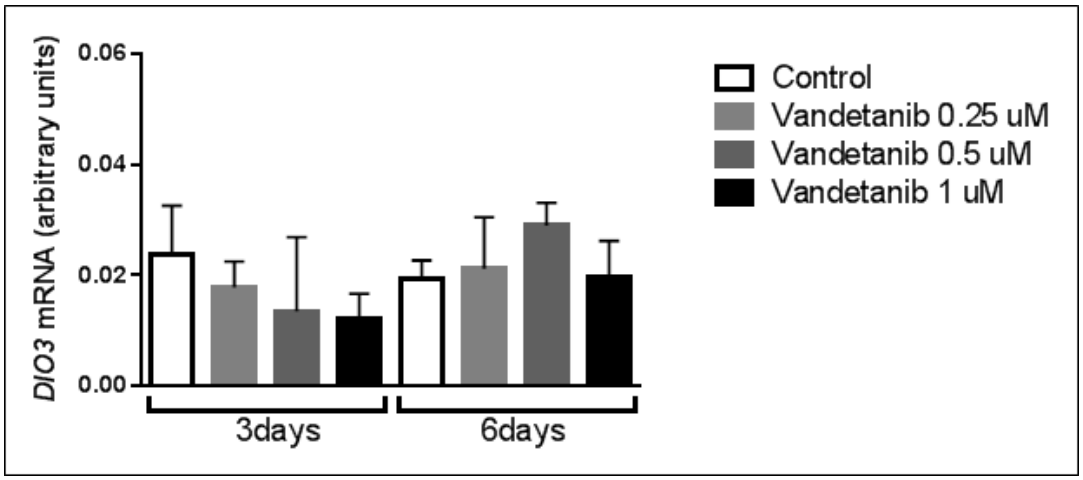

Figure 3: Effect of vandetanib on *DIO3* mRNA expression. Cells were treated with increasing concentrations of vandetanib (0.25, 0.5 and 1 µM) for 3 and 6 days. Vandetanib did not increase *DIO3* mRNA levels after 3 or 6 days of treatment (*P*=0.34 and *P*=0.33, respectively).

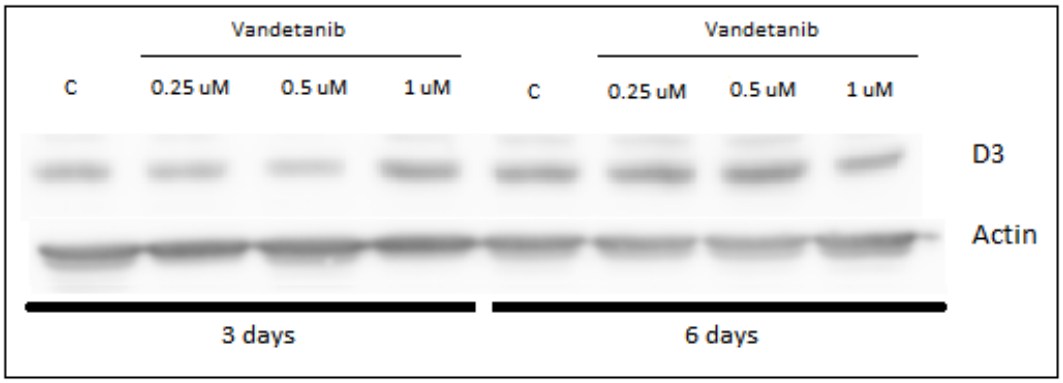

Figure 4: Effect of vandetanib on D3 protein levels. Cells were treated with different concentrations of vandetanib; 30 ug of total cells lysates were immunoblotted with anti-D3 antibody. The membrane was stripped and reprobed with β-actin, used for normalization. Vandetanib did not increase D3 protein levels after 3 or 6 days of treatment.

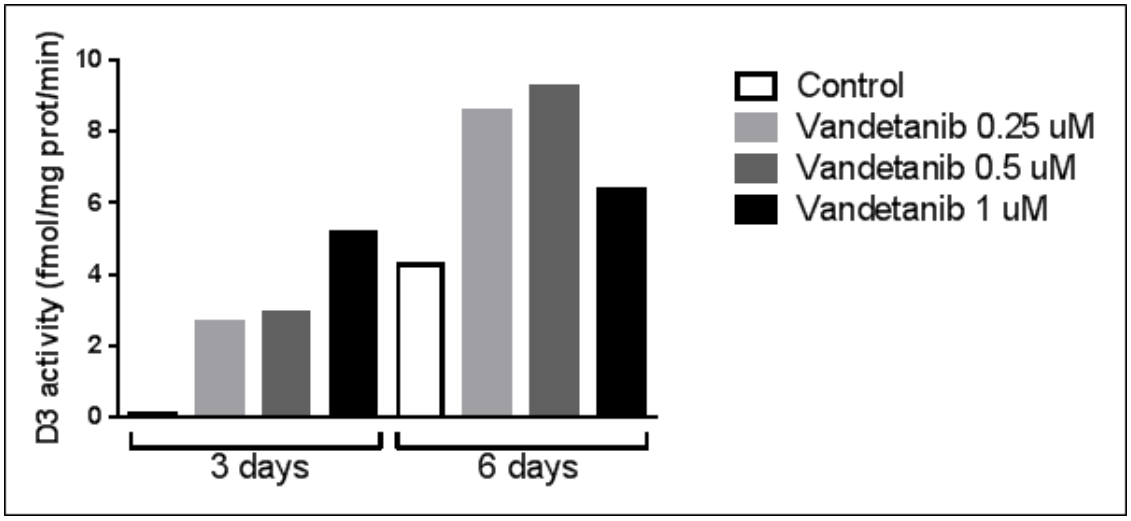

Figure 5: Effect of vandetanib on D3 activity. Vandetanib induced a 2 to 5-fold increase in the treated groups after 3 days of treatment and a 1.5 to 2.15-fold increase after 6 days of treatment. Data are representative of two experiments.