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1,25-Dihydroxyvitamin D_3 and a superagonistic analog in combination with paclitaxel or suberoylanilide hydroxamic acid have potent antiproliferative effects on anaplastic thyroid cancer

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ABSTRACT

Anaplastic thyroid cancer represents one of the most aggressive cancers. The active form of vitamin D, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), has been shown to have antiproliferative and/or redifferentiating properties in several malignancies, including thyroid cancer. The objective of this study was to investigate the effects of $1,25(OH)_2D_3$ and the superagonistic analog CD578 in anaplastic thyroid cancer, alone or in combination with paclitaxel, a taxane, and suberoylanilide hydroxamic acid (SAHA), a potent histone deacetylase inhibitor with promising effects in undifferentiated thyroid cancer.

Four human thyroid cancer cell lines (FTC-133, C643, 8505C and HTh74) were treated with $1,25(OH)_2D_3$ or CD578, alone or in combination with paclitaxel or SAHA. Effects on cell growth and differentiation were evaluated.

Clear effects on growth arrest were observed in a clonogenic assay, and absolute cell counts demonstrated a 24–36% reduction in all cell lines after 72 h treatment with $1,25(OH)_2D_3$ (10^{-6} M) and a 60% inhibition after 120 h in the most sensitive cell line HTh74. A similar growth inhibition was shown after treatment with a 1000-fold lower concentration of analog CD578. This growth arrest was explained by antiproliferative effects, further supported by an increased % of cells in the G_0–G_1 phase of the cell cycle and by a decreased transcription factor E2F1 mRNA expression. Combination treatments of $1,25(OH)_2D_3$ or CD578 with paclitaxel or SAHA resulted in an additive and in some conditions a synergistic effect on the inhibition of proliferation. Redifferentiation analysis revealed only a modest increase in sodium iodide symporter and thyroglobulin mRNA expression after treatment with $1,25(OH)_2D_3$, without additive effect after combination treatment. No effects were observed on TSH-receptor or thyroid peroxidase mRNA expression.

Our *in vitro* findings demonstrate that the superagonistic vitamin D analog CD578 holds promise as adjuvant antiproliferative therapy of anaplastic thyroid cancer, especially in combination with other drugs such as paclitaxel or SAHA.

1. Introduction

Anaplastic thyroid cancer is characterized by very aggressive growth and loss of expression of sodium-iodide symporter (NIS) and TSH-receptor (TSH-R), leading to failure of conventional therapy, consisting of thyroidectomy, adjuvant treatment with radioactive iodine and TSH-suppressive thyroid replacement therapy [1,2]. Despite the use of multimodality therapies combining surgery, radiotherapy and aggressive chemotherapeutic regimens, survival rates remain extremely poor. Therefore, the major challenge in the treatment of anaplastic thyroid cancer consists in the development of new agents capable of inhibiting tumor growth and/or inducing redifferentiation, and with more efficacy and/or less cytotoxicity when used in combination with the current chemotherapeutics. In the past years several antiproliferative and redifferentiating agents for thyroid cancer have been described, such as retinoids, PPAR-γ agonists, histone deacetylase inhibitors [3] and also 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$].

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$1,25(OH)_2D_3$, the active form of vitamin D, is well known for its role in bone and mineral homeostasis. It exerts its genomic action through binding with the nuclear vitamin D receptor (VDR), a member of the steroid hormone receptor superfamily of ligand-activated transcription factors. In recent years, we and others have demonstrated interesting antiproliferative and redifferentiating properties of $1,25(OH)_2D_3$ in several malignant cell types [4,5]. Also in models of thyroid cancer, $1,25(OH)_2D_3$ has been shown to induce growth arrest by increasing the expression of cell cycle inhibitor p27, to restore thyroglobulin (Tg) expression in tumors of immune-deficient mice xenografted with WRO cells (representing human well-differentiated thyroid follicular cancer) and to increase cell adhesion by increasing fibronectin expression [6–8]. Furthermore VDR polymorphisms have been associated with thyroid cancer, which may modify the risk of cancer [9,10]. In human papillary thyroid cancer tissue it has been shown that the expression of VDR and 1α-hydroxylase, the enzyme responsible for the final hydroxylation step from 25-OHD_3 towards $1,25(OH)_2D_3$, is increased, suggesting a sensitivity and a role for $1,25(OH)_2D_3$ in the neoplastic thyroid [11]. In the meantime numerous structural analogs of $1,25(OH)_2D_3$ have been designed with a spectrum of selectivity profiles, promising in the treatment of neoplastic disorders [12]. For the present study the nonsteroidal 17-methyl D-ring analog CD578 was selected. This compound has been shown to be 20-fold more potent in the inhibition of proliferation of MCF-7 breast cancer cells and to be 100-fold less calcemic *in vivo* compared to the parent compound [13,14].

The objective of the present study was to further investigate the antiproliferative and prodifferentiating effects of $1,25(OH)_2D_3$ and analog CD578 in thyroid cancer. Four human thyroid cancer cell lines were used, representing poorly differentiated to anaplastic thyroid cancer. Furthermore, the effects of combination therapy were studied with paclitaxel, a cytotoxic chemotherapeutic agent from the taxane family clinically used in anaplastic thyroid cancer [15,16], and with suberoylanilide hydroxamic acid (SAHA), a member of the histone deacetylase inhibitor family, with promising effects on thyroid cancer [17,18]. The combination of $1,25(OH)_2D_3$ or CD578 with paclitaxel or SAHA resulted in additive or synergistic effects on growth inhibition. This extra effect can lead to the use of lower doses of chemotherapeutics and therefore less toxicity.

2. Materials and methods

2.1. Reagents

$1,25(OH)_2D_3$ was a generous gift from J.P. van de velde (Solvay, Weesp, Netherlands). CD578, a 17-methyl-19-nor-21-nor-23-yne-26,27-F6-$1,25(OH)_2D_3$-ring analog has been described previously [13]. Paclitaxel (Taxol®) was purchased from Bristol–Myers Squibb Belgium (Brussels, Belgium) and SAHA was purchased from Cayman (Tallin, Estonia).

2.2. Cell culture

The human anaplastic HTh74 and C643 cell line were generously donated by Dr N.-E. Heldin (Uppsala University, Uppsala, Sweden). The FTC-133 cell line was kindly provided by Dr C. Schmutzler (Institut für Experimentelle Endokrinologie, Charité – Universitätsmedizin Berlin, Berlin, Germany) and represents more differentiated follicular thyroid cancer cells. The undifferentiated thyroid carcinoma 8505C cell line was purchased from the European Collection of Cell Cultures (ECACC) (Salisbury, UK). All cell lines were maintained in RPMI1640 + glutaMax™ (HTh74, C643, 8505C) or DMEM/F12 (FTC-133) media, supplemented with 10% FCS (5% for 8505C), 1% penicillin/streptomycin (10,000 U/mL and 10,000 μg/mL, respectively), all purchased from Invitrogen (Merelbeke, Belgium). All cell lines have been genetically profiled (UCCC DNA Sequencing & Analysis Core, Denver, USA) by Short Tandem Repeat analysis and are consistent with their previously published profiles [19–22].

2.3. Count experiments

Cells were seeded in 75 cm^2 culture flasks at a density of 100,000/50,000 cells/flask (HTh74, FTC-133), 250,000/150,000 (C643) and 50,000/25,000 cells/flask (8505C) for respectively the 72 h/120 h time point. Two days after seeding the cell lines were treated with a single dose of $1,25(OH)_2D_3$ (10^{-6} M), paclitaxel (3×10^{-9} M) or SAHA (10^{-7} M), ethanol (vehicle) or a combination of $1,25(OH)_2D_3$ and paclitaxel or SAHA. Concentrations approaching EC50 values in pilot proliferation experiments were chosen for paclitaxel and SAHA. Cells were harvested by trypsin digestion (Invitrogen) and thoroughly resuspended in 5 mL medium. 0.1 mL trypan blue was added to an aliquot (0.4 mL) of this cell suspension and trypan blue-negative cells were counted using a Burker chamber. Per experiment duplicate samples were counted.

2.4. Clonogenic assay

FTC-133 and HTh74 (both 250 cells/well), 8505C (500 cells/well) and C643 (2000 cells/well) cells were seeded in 6-well plates. After 2 days cells were incubated with a single dose of $1,25(OH)_2D_3$ (10^{-6} M) or ethanol (vehicle). In a separate experiment HTh74 cells were also treated with 10^{-6} M, 10^{-8} M, and 10^{-9} M $1,25(OH)_2D_3$ and 10^{-6} M and 10^{-9} M CD578. Colony formation was monitored microscopically. On day 10 cells were washed with PBS, fixed with paraformaldehyde (4%) and stained with 0.1% crystal violet (Sigma–Aldrich) in 25% methanol. Colonies >0.5 mm diameter were counted. Two experiments containing duplicate samples were performed.

2.5. Proliferation assay

8505C, FTC-133, HTh74 and C643 cells were seeded at a density of 2000–3000 cells/well in a 96-well microtiter plate (M.L.S. N.V., Menen, Belgium). After a 2 day pre-incubation period, cells were treated with $1,25(OH)_2D_3$ (4×10^{-10} M to 3.125×10^{-5} M), alone or in combination with paclitaxel (2.5×10^{-10} M to 3.2×10^{-8} M) or SAHA (5×10^{-8} M to 6.4×10^{-6} M) and ethanol (vehicle). HTh74 cells and FTC-133 cells were also treated with CD578 (2×10^{-9} M to 6.25×10^{-6} M) alone or in combination with respectively paclitaxel (5×10^{-10} M to 1.6×10^{-8} M) or SAHA (10^{-7} M to 3.2×10^{-6} M). After 3 days cell proliferation was assessed by measuring [^{3}H]-thymidine incorporation. During 4 h cells were incubated with 1 μCi [^{3}H]-thymidine (specific activity of 2.0 or 20–40 Ci/mmol) (MP Biomedicals Europe, Elsene, Belgium). Then cells were harvested on filter paper (Unifilter-96 GF/C, Perkin Elmer, Zaventem, Belgium) by a semi-automatic cell harvesting system. Finally, radioactivity was measured in a microplate scintillation counter (Topcount, Packard Instrument Company, Meriden, USA). Each time point and treatment condition was carried out in 5-fold and each experiment was repeated at least 2 times.

2.6. Cell cycle analysis

Two days after seeding (same conditions as cell count experiments) the four cell lines were incubated with $1,25(OH)_2D_3$ (10^{-6} M), alone or in combination with paclitaxel (3×10^{-9} M) or SAHA (10^{-7} M) and ethanol (vehicle). Three days later cells were harvested, washed twice with PBS and fixated with ice-cold 70% ethanol (100,000 cells/sample). After 20 min incubation on

ice, the cells were washed twice with PBS/Tween®20 and resuspended in PBS/Tween®20 with 0.5 mg/mL propidium iodide and 1 mg/mL RNase A (Sigma–Aldrich). Finally samples were analyzed with a FACSort flow cytometer (Becton Dickinson, Erembodegem, Belgium). Per experiment, duplicate samples were performed.

2.7. Quantitative real-time RT-PCR analysis

FTC-133, HTh74, C643 and 8505C cells were plated out and left untreated for 2 days. Cells were collected 24 h and 72 h after treatment with $1,25(OH)_2D_3$ (10^{-6} M), paclitaxel (2×10^{-9} M) or SAHA (10^{-7} M) or a combination of $1,25(OH)_2D_3$ and paclitaxel/SAHA and ethanol (vehicle). Total RNA was isolated by the RNeasy® mini protocol and treated with RNase-Free DNase (Qiagen Benelux, Venlo, Netherlands). Using Superscript II RT (Invitrogen) 1 μg RNA was reverse transcribed at 42 °C for 80 min in the presence of 5 μM oligo(dT) (Roche, Vilvoorde, Belgium). PCR analysis (MyIQ Single Color Real Time PCR Detection System, Bio-Rad Laboratories Nazareth, Belgium) was carried out as previously described [23]. Each experiment was run in triplicate. Primer and probe sequences (Eurogentec, Seraing, Belgium) for E2F1, Ki67, p21, 24-hydroxylase, β-actin, NIS, thyroid peroxidase (TPO), TSH-R and Tg are available upon request. All real-time RT-PCR were performed with dual-labeled fluorescent Taqman® probes, with the exception of Tg, where Sybr green was used (Eurogentec). External controls were constructed consisting of plasmid standard curves, each containing a known amount of input copy number. Two experiments containing duplicate samples were performed.

2.8. Statistical analysis

Statistical analysis was performed using the software program Statistica (StatSoft, Inc, Tulsa, USA). Results are expressed as mean ± SD of three independent experiments, except otherwise stated. The unpaired Student's *t* test and ANOVA test were performed where appropriate. When the ANOVA was significant, the Fisher's LSD multiple-comparison test was applied. Significance was defined at the 0.05 level. For the [^{3}H]-thymidine incorporation a syngenicity test based on the median effect principle of Chou and Talalay was used [24]. Briefly, the median effect principle stated that the interaction (synergism, additivity, antagonism) between 2 agents can be quantified using the combination index CI_x:

$$CI_x = \frac{ED_x{}^{1c}}{ED_x{}^{1a}} + \frac{ED_x{}^{2c}}{ED_x{}^{2a}}$$

where ED_x was the dose of the 1st/2nd agent alone (a) or in combination (c) needed to produce a given effect level *x*. The agents are synergistic, additive or antagonistic when $CI_x < 1$, =1 or >1, respectively. Here, the effect considered was the inhibition of proliferation measured by [^{3}H]-thymidine at the 50% effect level, thus 50% proliferation inhibition.

3. Results

3.1. Effect of $1,25(OH)_2D_3$ and CD578 on thyroid cancer cell growth

Absolute cell count numbers gave a first indication of the effect of $1,25(OH)_2D_3$ on thyroid cancer cells after 72 h or 120 h treatment with 10^{-6} M $1,25(OH)_2D_3$. A 24–29% inhibition was achieved in three cell lines (FTC-133, C643, 8505C) after 72 h incubation. Cell counts were not further reduced after 120 h incubation with $1,25(OH)_2D_3$. With 36% and 60% cell reduction after respectively 72 h and 120 h ($p < 0.02$), the fourth cell line HTh74 was the most responsive to $1,25(OH)_2D_3$ (Fig. 1A). The growth-inhibitory effect of $1,25(OH)_2D_3$ on thyroid cancer cells was confirmed by a clonogenic assay. In all four thyroid cancer cell lines, smaller and/or fewer colonies were formed after incubation for at least 10 days with a single dose of 10^{-6} M $1,25(OH)_2D_3$. Treatment with analog CD578 at a 1000-fold lower concentration equally reduced the number and size of colonies formed in HTh74 cells (Fig. 1B), while this effect was almost completely lost treating the cells with 10^{-9} M $1,25(OH)_2D_3$.

The decreased cell numbers were accompanied by inhibition of cell proliferation measured by [^{3}H]-thymidine incorporation (Fig. 2A, left panel). The cell lines were treated with $1,25(OH)_2D_3$ in a concentration range from 4×10^{-10} M to 3.125×10^{-5} M and incubated for 72 h. Again the FTC-133, C643 and 8505C cell lines shared a similar dose–response profile to $1,25(OH)_2D_3$. At 1.25×10^{-6} M $1,25(OH)_2D_3$ (the concentration closest to 10^{-6} M used in all other experiments) these cell lines showed a 10–17% proliferation inhibition. The HTh74 cell line was the most sensitive to $1,25(OH)_2D_3$ in the whole concentration range. A 49% proliferation inhibition was seen at the $1,25 \times 10^{-6}$ M concentration, but a significant 23% inhibition was already observed at the much lower concentration of 2×10^{-9} M. FTC-133 and HTh74 cells were also treated with analog CD578 for 72 h (Fig. 2A, right panel). At high concentrations a similar inhibition of HTh74 cell proliferation was induced by $1,25(OH)_2D_3$ and CD578. However, at low concentrations (10^{-9} M), CD578 induced a 2-fold stronger proliferation inhibition in the HTh74 cells compared to $1,25(OH)_2D_3$ (43% versus 24%). To achieve a similar proliferation inhibition the analog CD578 could thus be used at lower concentrations compared to $1,25(OH)_2D_3$. Although not significant, CD578 induced a small inhibition of proliferation of FTC-133 cells, even at 10^{-9} M.

For the most sensitive cells to $1,25(OH)_2D_3$ (HTh74) cell cycle distribution demonstrated a clear increase of cells in the G_0–G_1 phase (from 55% to 67%), accompanied by a decrease of cells in the G_2–M phase (from 19% to 15%) after a 72 h incubation period with 10^{-6} M $1,25(OH)_2D_3$ ($p < 0.02$). A similar, but small response to $1,25(OH)_2D_3$ in the G_0–G_1 phase was observed for the FTC-133 and 8505C cells, although a decrease in G_2–M phase was absent. For the least responsive cell line C643 no clear increase in G_0–G_1 or decrease in G_2–M phase could be detected (data not shown).

The expression of the transcription factor E2F1, known to be involved in proliferation and cell cycle progression, was investigated after 24 h and 72 h incubation with 10^{-6} M $1,25(OH)_2D_3$. The earlier time point of 24 h was chosen since early effects of $1,25(OH)_2D_3$ on mRNA expression of proliferation-related genes are reported. E2F1 decreased after 24 h incubation with $1,25(OH)_2D_3$ in 8505C cells and after 72 h in all cell lines (although not significantly in the FTC-133 and HTh74 cell line). No change occurred in the expression of p21 after treatment with $1,25(OH)_2D_3$. The proliferation inhibition was further supported through a decreased expression of the proliferation marker Ki67 after 24 h and 72 h in all cell lines (Fig. 2B).

3.2. Antiproliferative effect of combination treatment with paclitaxel

To investigate the effect of the combination with other anticancerous agents, $1,25(OH)_2D_3$ was first combined with the cytotoxic chemotherapeutic paclitaxel. Absolute cell count numbers were collected after 72 h and 120 h incubation with 10^{-6} M $1,25(OH)_2D_3$ and/or 3×10^{-9} M paclitaxel. Monotherapy with paclitaxel demonstrated a clear effect on cell numbers, being reduced with 44–78% after 72 h (Fig. 3A) with 77–95% after 120 h incubation. The combination of $1,25(OH)_2D_3$ and paclitaxel led to a small additional reduction in cell counts of 1–12% compared to monotreatment paclitaxel in FTC-133, C643 and 8505C cells.

Through [^{3}H]-thymidine incorporation the antiproliferative effect of $1,25(OH)_2D_3$ combined with paclitaxel in a range

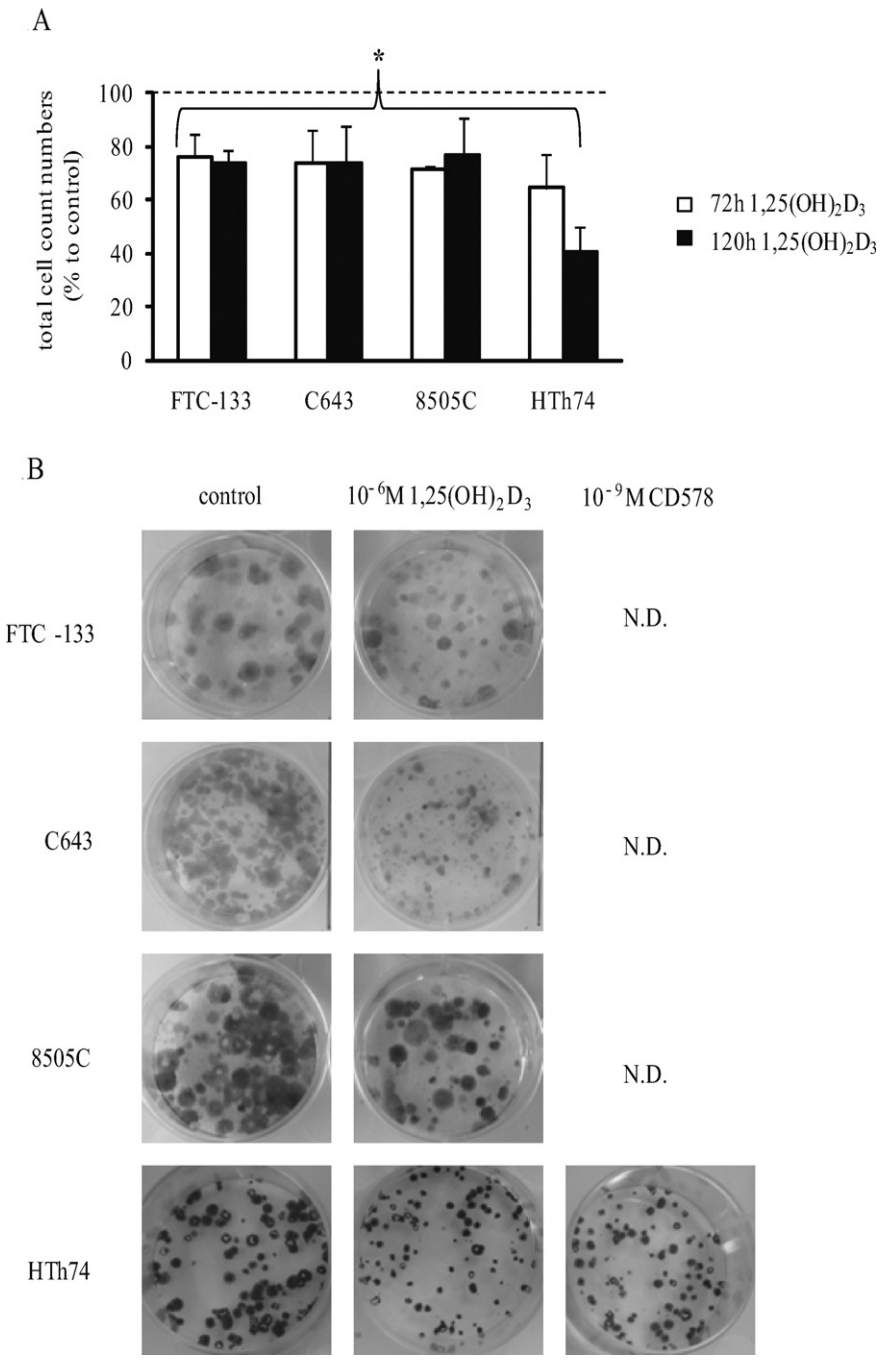

Fig. 1. Effect of $1,25(OH)_2D_3$/analog on thyroid cancer cell growth. (A) Effect of $1,25(OH)_2D_3$ on absolute cell count numbers. FTC-133, C643, 8505C and HTh74 cells were treated for 72 h or 120 h with 10^{-6} M $1,25(OH)_2D_3$ or vehicle (control). $^*p < 0.05$ compared to control (100%) at same time point. (B) Effect of $1,25(OH)_2D_3$ and CD578 on colony formation by means of a clonogenic assay. After at least 10 days of treatment the formed colonies were photographed (one representative experiment is shown).

of concentrations was investigated. Monotherapy of 3×10^{-9} M paclitaxel (concentration used in the cell count experiment) demonstrated a 48–69% proliferation inhibition after 72 h incubation in the four cell lines ($p < 7 \times 10^{-6}$). Combination of paclitaxel with $1,25(OH)_2D_3$ led to an additional proliferation inhibition in FTC-133 and C643 cells in all investigated concentration combinations (Fig. 3B) and in a few combinations in 8505C and HTh74 cells. In order to elucidate true synergistic responses between paclitaxel and $1,25(OH)_2D_3$ the combination index (CI) according to Chou and Talalay was calculated for 50% proliferation inhibition. Based on CI values <1, the co-treatment of paclitaxel with $1,25(OH)_2D_3$ was synergistic for FTC-133 (CI = 0.77) and C643 (CI = 0.72) cells. The experiment was repeated with paclitaxel and analog CD578 in FTC-133 cells, confirming additional proliferation inhibition also at lower concentrations of CD578 (Fig. 3C). Moreover, synergism was confirmed (CI = 0.86).

3.3. Antiproliferative effect of combination treatment with SAHA

After a 72 h incubation period with 10^{-7} M SAHA all four cell lines demonstrated a reduction in cell count numbers, ranging from 19% to 29% reduction (Fig. 4A), which was significant for 8505C and HTh74 cells. Next, SAHA (10^{-7} M) was combined with $1,25(OH)_2D_3$ (10^{-6} M), leading to a 6–12% added reduction in absolute cell count numbers in FTC-133, 8505C and HTh74 cells after 72 h compared to

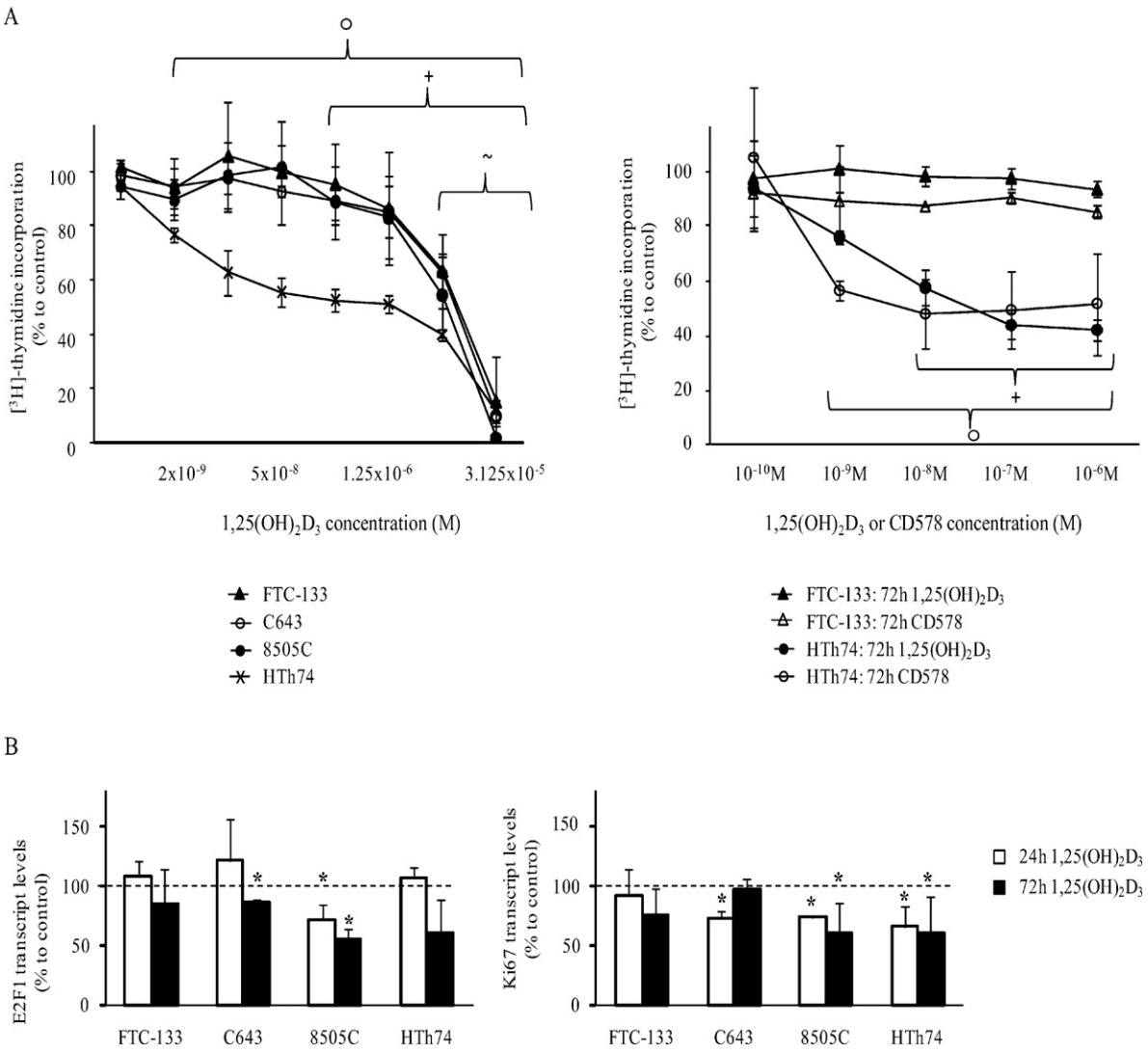

Fig. 2. Effect of $1,25(OH)_2D_3$/analog on thyroid cancer cell proliferation. (A, left panel) Proliferation inhibition of FTC-133, C643, 8505C and HTh74 cells, treated with increasing concentrations of $1,25(OH)_2D_3$ or vehicle. After 72 h of treatment [^{3}H]-thymidine incorporation was assessed and expressed relative to uptake in control cultures (100%). $p < 0.0004$ compared to control for FTC-133 and 8505C (∼), $p < 0.05$ compared to control for C643 (+) and $p < 2 \times 10^{-8}$ for HTh74 (○). (Right panel) Proliferation inhibition of FTC-133 and HTh74 cells after 72 h treatment with $1,25(OH)_2D_3$, CD578 or vehicle. $p < 0.004$ compared to control for HTh74 cells treated with $1,25(OH)_2D_3$ (+) or CD578 (○). (B) mRNA expression of E2F1 and Ki67 in thyroid cancer cells as measured by real-time RT-PCR. After 24 h and 72 h treatment with $1,25(OH)_2D_3$ (10^{-6} M) or vehicle, total RNA was harvested. Gene expression was normalized to β-actin mRNA levels and expressed as a ratio between $1,25(OH)_2D_3$-treated samples and corresponding control samples. *$p < 0.03$ compared to control.

monotherapy. In the same three cell lines, an additional 4–22% cell count reduction was observed after 120 h of incubation compared to monotherapy.

[^{3}H]-thymidine incorporation demonstrated a 16–20% ($p < 0.007$) proliferation inhibition with mono-treatment of 10^{-7} M SAHA (concentration used in the cell count experiment) in 8505C and HTh74 cells. Higher SAHA concentration (1.6×10^{-6} M) substantially increased the proliferation inhibition to 77%, 80% and 92% in respectively FTC-133, 8505C and HTh74 cells, but only to 19% in the C643 cells ($p < 0.00002$), which is in line with the lack of response of C643 cells to longer incubation periods (120 h) in the cell count experiment. There was an additive effect for all thyroid cancer cell lines at higher concentrations of $1,25(OH)_2D_3$ and SAHA, while for HTh74 this extra effect on proliferation inhibition could also be seen at lower concentrations of both agents (Fig. 4B). True synergy was found according to Chou and Talalay for co-treatment of $1,25(OH)_2D_3$ and SAHA (CI = 0.85) in HTh74 cells. Finally, HTh74 cells were treated with CD578 in combination with SAHA for 72 h (Fig. 4C). Again an additive effect was observed when both agents were combined. Furthermore, this effect was synergistic (CI = 0.70).

3.4. Prodifferentiating effect of $1,25(OH)_2D_3$ and the combination with paclitaxel or SAHA

In order to assess the potential effects of $1,25(OH)_2D_3$ on redifferentiation of thyroid cancer cells, the expression of a panel of thyroid-specific genes was studied: NIS, Tg, TPO, and TSH-R. For NIS, there was a trend, although very modest, towards increased expression in FTC-133 and HTh74 cells (Fig. 5A, left panel). For Tg there was a minor 1.4–1.7-fold increased mRNA expression after 24 h in FTC-133, 8505C and HTh74 cells and after 72 h for HTh74 cells (Fig. 5A, right panel). Expression of TPO and TSH-R was undetectable in non-treated cells of all four cell lines and remained undetectable after incubation with 10^{-6} M $1,25(OH)_2D_3$.

Monotreatment with paclitaxel increased NIS mRNA expression in all four cell lines after 24 h (2–3-fold) and 72 h (1.8–8-fold) as well as Tg mRNA expression (1.8–8.1-fold and 2.5–8.9-fold, respectively

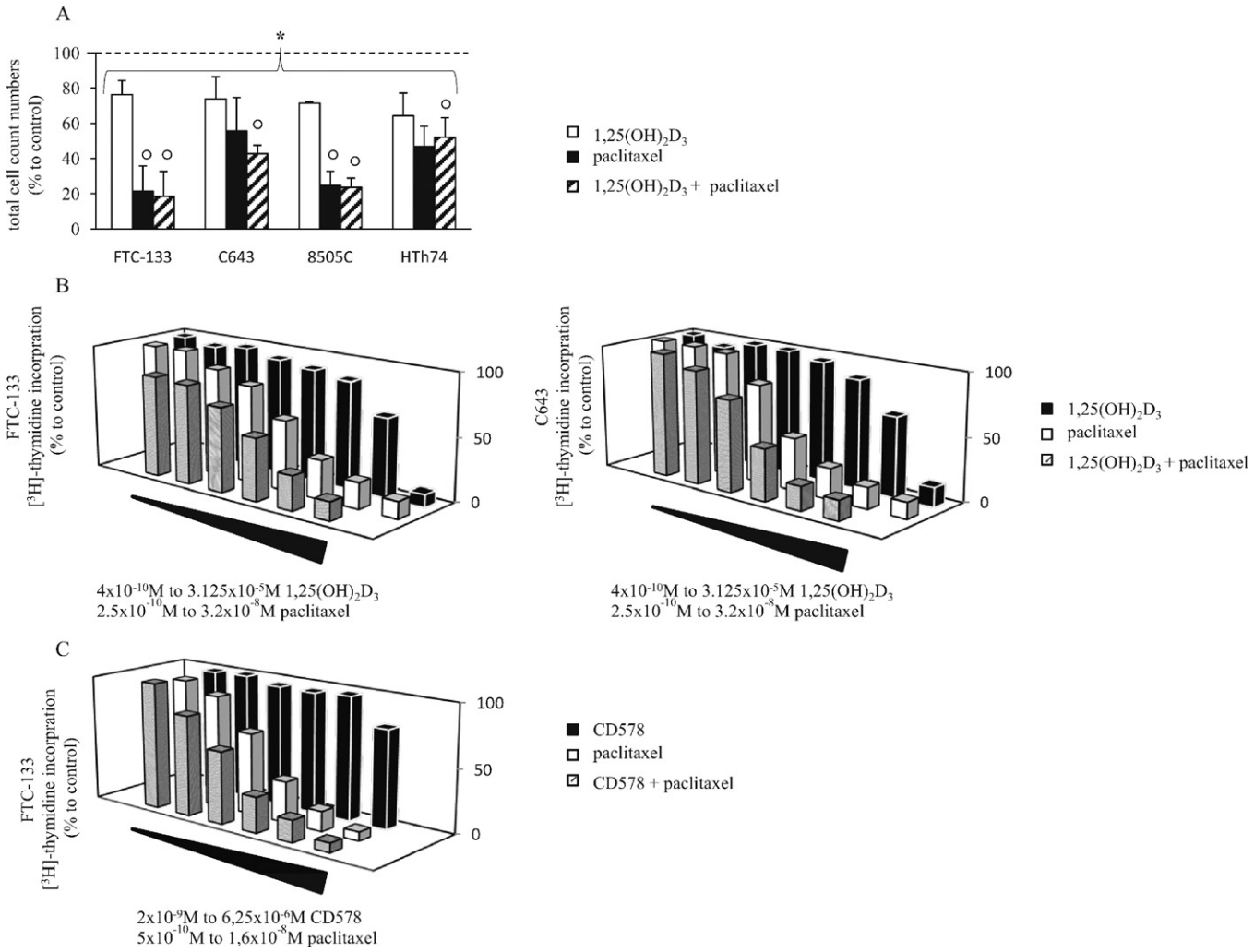

Fig. 3. Effect of $1,25(OH)_2D_3$/analog combined with paclitaxel on thyroid cancer cell growth and proliferation. (A) FTC-133, C643, 8505C and HTh74 cells were treated with 10^{-6} M $1,25(OH)_2D_3$, 3×10^{-9} M paclitaxel, their combination or vehicle for 72 h. $^*p < 0.03$ compared to control (100%); (○) $p < 0.05$ compared to $1,25(OH)_2D_3$. (B) Proliferation inhibition ([^{3}H]-thymidine incorporation) of FTC-133 and C643 cells after 72 h of treatment with a concentration range of $1,25(OH)_2D_3$, paclitaxel or their combination. (C) Proliferation inhibition ([^{3}H]-thymidine incorporation) of FTC-133 cells after 72 h of treatment with a concentration range of CD578, paclitaxel or their combination.

after 24 and 72 h) (Fig. 5B). Monotreatment with SAHA only modestly increased NIS expression and only in HTh74 cells (2-fold and 1.7-fold at 24 h and 72 h, respectively, $p < 0.05$), whereas Tg expression levels were not influenced in any cell line. The combination of paclitaxel or SAHA with $1,25(OH)_2D_3$ did not result in additional effects on NIS or Tg expression levels (data not shown).

4. Discussion

This study demonstrates the antiproliferative effects of $1,25(OH)_2D_3$, the superagonistic vitamin D analog CD578, paclitaxel and SAHA on anaplastic thyroid cancer cells, and the augmentation of antiproliferative effects in combination with paclitaxel and SAHA, two mechanistically different agents. The potential of $1,25(OH)_2D_3$ for the treatment of diverse cancer cell types is acknowledged since decades. We and others have shown that important cell cycle regulators are involved such as cyclins, cyclin-dependent kinases and their corresponding inhibitors as well as E2F transcription factors and accompanying pocket proteins [4,5,25].

We first confirmed the significant effects of $1,25(OH)_2D_3$ on growth arrest of anaplastic thyroid cancer, as previously shown by others [6,26]. VDR expression has been demonstrated in normal thyroid tissue, papillary thyroid cancer and medullary thyroid cancer [11,27,28]. The induction of 24-hydroxylase mRNA expression, a known highly induced target gene of $1,25(OH)_2D_3$, verified the sensitivity of the thyroid cancer cell lines used in the present study for $1,25(OH)_2D_3$ (data not shown). After treatment with $1,25(OH)_2D_3$, growth arrest was accompanied by an accumulation of cells in the G_1-phase, but only in the thyroid cancer cell lines which were most responsive to $1,25(OH)_2D_3$. Furthermore, clear inhibition of proliferation was observed. The absence of any effect of $1,25(OH)_2D_3$ on apoptosis of thyroid cancer cells, as shown by others [6], suggested that growth arrest was solely due to antiproliferative effects. Calcemic side effects at high concentrations prevent the clinical application of $1,25(OH)_2D_3$ as an antiproliferative agent. However, a range of structural analogs has been designed with discrepant antiproliferative and calcemic effects. In the present study the superagonistic nonsteroidal 17-methyl D-ring analog CD578 with low calcemic effects was used, and confirmed clear antiproliferative effects at low, clinically achievable, concentrations.

Gene expression analysis demonstrated reduced expression of Ki67, a gene expressed in all phases of the cell cycle (except G_0) and used as a general proliferation marker, frequently found in aggressive cancers and shown to be proportional to tumor grade

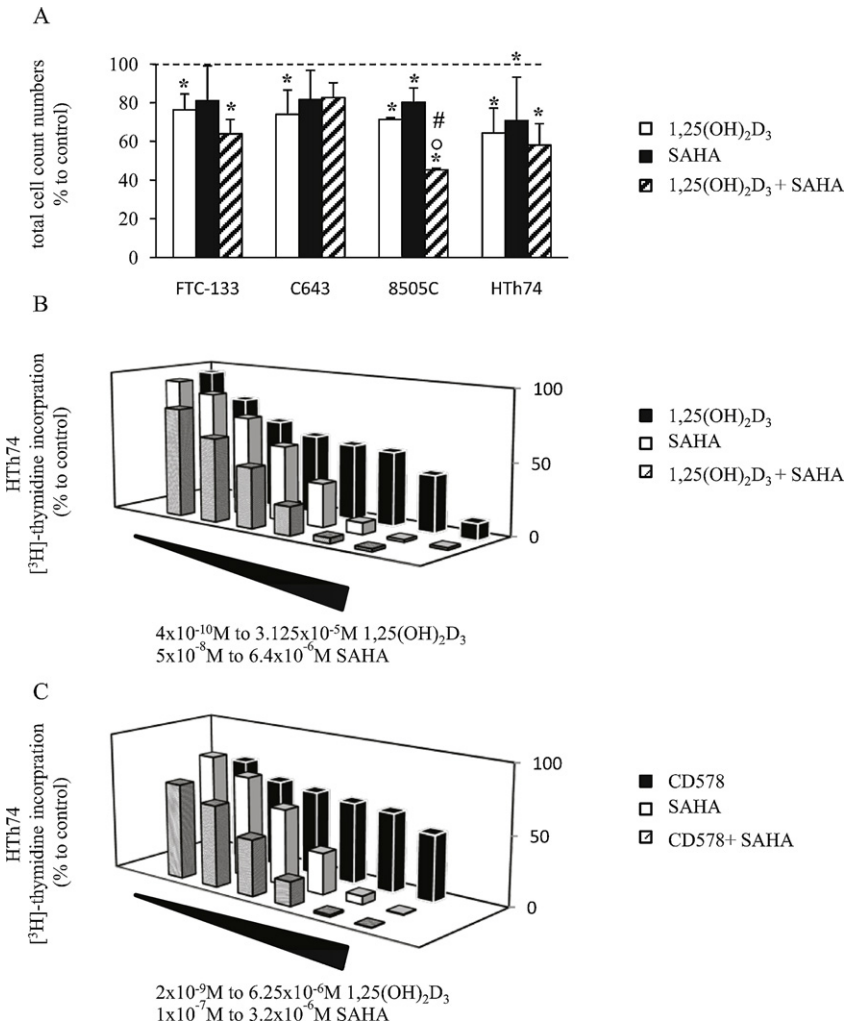

Fig. 4. Effect of $1,25(OH)_2D_3$/analog combined with SAHA on thyroid cancer cell growth and proliferation. (A) Effect of $1,25(OH)_2D_3$ on absolute cell count numbers. FTC-133, C643, 8505C and HTh74 cells were treated with 10^{-6} M $1,25(OH)_2D_3$, 10^{-7} M SAHA, their combination or vehicle for 72 h. $^*p < 0.02$ compared to control (100%); (○) $p < 0.0006$ compared to $1,25(OH)_2D_3$ and $^\#p < 0.0002$ compared to SAHA. (B) Proliferation inhibition ([^{3}H]-thymidine incorporation) of HTh74 cells after 72 h of treatment with a concentration range of $1,25(OH)_2D_3$, SAHA or their combination. (C) Proliferation inhibition ([^{3}H]-thymidine incorporation) of HTh74 cells after 72 h of treatment with a concentration range of CD578, SAHA or their combination.

and number of mitoses in papillary thyroid cancer [29,30]. Furthermore, analysis of genes involved in cell cycle progression showed a reduced expression of E2F1, a transcription factor with target genes involved in DNA replication and cell cycle progression from the G_1 to S phase. The degree of decreased E2F1 expression was not completely consistent with the sensitivity of the different cell lines to the antiproliferative effect induced by $1,25(OH)_2D_3$. However $1,25(OH)_2D_3$ exerts its growth inhibitory effect through a wide array of transcription factors, besides E2F1 and a more prominent role for other genes is probable in different thyroid cancer cell lines [31]. We did not observe an altered expression level of the cyclin dependent kinase p21 after $1,25(OH)_2D_3$ treatment. Liu et al. demonstrated an accumulation of p27, another cyclin dependent kinase inhibitor, in thyroid cancer cells treated with $1,25(OH)_2D_3$ [6].

Dose limitation due to serious side effects represents a drawback for antiproliferative agents used for treatment of anaplastic cancer. Combination treatments with different antineoplastic agents at tolerable doses might provide a solution. Paclitaxel, member of the taxane family, represents a microtubule-disrupting agent and is characterized by mitotic block and apoptosis induction. In a prospective phase II clinical trial significant but limited clinical activity was demonstrated in patients with persistent or metastatic anaplastic thyroid cancer after treatment with paclitaxel. Peripheral neuropathy was a major side effect [16]. In another promising clinical trial a 33% response rate with long term survival was seen for anaplastic thyroid cancer patients in stage IVB who underwent induction chemotherapy with weekly paclitaxel. Stage IVC patients had a significant clinical benefit, but without improved survival [32]. In other studies, co-treatment with $1,25(OH)_2D_3$ and paclitaxel resulted in enhanced anti-tumor activity in models of prostate cancer and breast cancer [33,34]. A clinical pharmacokinetic study of the combination treatment even suggested attenuation of the $1,25(OH)_2D_3$-induced-hypercalcemia in combination with paclitaxel [35]. In the present study, we demonstrate for the first time additional growth retardation using combined treatment of paclitaxel and $1,25(OH)_2D_3$ in thyroid cancer cell lines. For two out of four cell lines the proliferation inhibition by the two agents was synergistic. Furthermore, a similar synergistic effect on proliferation inhibition was achieved through combination treatment with CD578 and paclitaxel in FTC-133 cells. A mechanism underlying this beneficial effect has not yet been uncovered. Hershberger et al. suggested that $1,25(OH)_2D_3$ decreased p21 expression in prostate cancer cells and squamous cell carcinoma (in contrast to increased expression in other tissues), and it is known that lowered p21 levels sensitize cells to paclitaxel-induced cell death [34]. How-

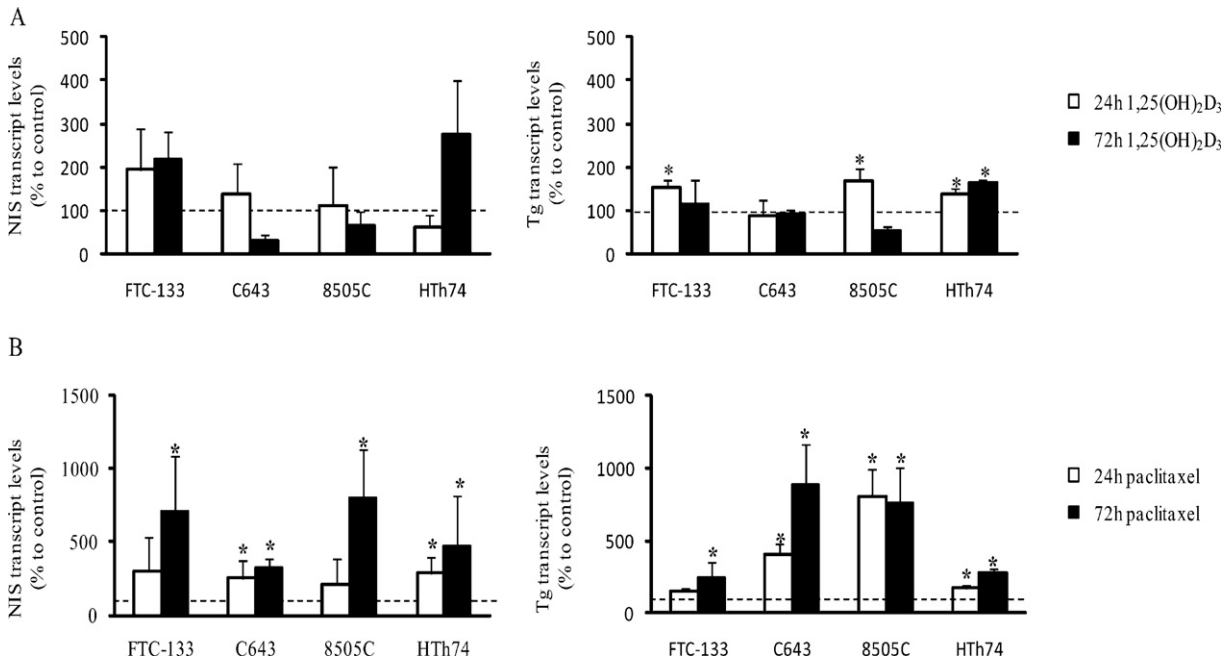

Fig. 5. Effect of $1,25(OH)_2D_3$ in mono and combined therapy on thyroid cancer cell differentiation. (A) mRNA expression of NIS and Tg in thyroid cancer cells as measured by real-time RT-PCR. After 24 h and 72 h treatment with $1,25(OH)_2D_3$ (10^{-6} M) or vehicle, total RNA was harvested. Gene expression was normalized to β-actin mRNA levels and expressed as a ratio between $1,25(OH)_2D_3$-treated samples and corresponding control samples. $^*p < 0.04$ compared to control. (B) mRNA expression of NIS and Tg after 24 h and 72 h incubation with 3×10^{-9} M paclitaxel. $^*p < 0.05$ compared to control.

ever in our thyroid cancer cells p21 expression levels were not altered.

Also the combination of $1,25(OH)_2D_3$ and SAHA was investigated. SAHA is a potent histone deacetylase inhibitor, with *in vitro* and *in vivo* antiproliferative and pro-apoptotic effects in anaplastic thyroid cancer [17,18,36]. Moreover, beneficial effects were observed in a phase I clinical trial in patients with advanced cancer, among which patients with thyroid cancer [37]. Major side effects were anorexia, dehydration, diarrhea and fatigue. Unfortunately a recent phase II trial with metastatic thyroid cancer showed neither complete nor partial response after SAHA treatment, with great adverse effects [38], but as the authors themselves suggested a dose alteration or combination treatment with another chemotherapeuticum might provide a solution. Our experiments confirmed the antiproliferative effect of SAHA on thyroid cancer cells, as shown *in vitro* and *in vivo* by others [17,18]. Moreover, we demonstrate additive and in several cell lines synergistic antiproliferative effects of SAHA combined with $1,25(OH)_2D_3$. Furthermore, the synergistic effect could be confirmed after treatment with CD578 and SAHA in HTh74 cells. Combination of SAHA with other antiproliferative agents may benefit from the more relaxed chromatin configuration caused by histone deacetylase inhibitors, which may enhance access to DNA by other antineoplastic agents, such as $1,25(OH)_2D_3$. Additionally, $1,25(OH)_2D_3$ corepressors possess histone deacetylase activity and keep chromatin in a closed, transcriptionally silent state. Banwell et al. hypothesized that combination of $1,25(OH)_2D_3$ with histone deacetylase inhibitors such as SAHA may help to overcome this transcriptional repression and increase VDR responsiveness [39].

Besides antiproliferative effects, we also investigated the potential of $1,25(OH)_2D_3$ in monotreatment and combination with paclitaxel/SAHA on redifferentiation. No change was observed in TSH-R or TPO expression after treatment with $1,25(OH)_2D_3$, but also at baseline (0 h) the cells expressed extremely low levels of TSH-R or TPO mRNA, suggesting their poorly differentiated to undifferentiated state. Akagi et al. showed a modest increase in NIS mRNA level after treatment with $1,25(OH)_2D_3$, a trend which we confirmed in the present study [36]. We showed only very mildly increased Tg expression. Also others did not observe increased Tg protein levels *in vitro* [6], but Dackiw et al. demonstrated increased Tg *in vivo* in immune-deficient mice xenografted with WRO cells [7]. Interestingly, monotreatment with paclitaxel substantially increased NIS and Tg expression levels in all four cell lines. Although the growth inhibiting effect of paclitaxel in different tumor types is well known, only one study mentions the redifferentiating capacity of paclitaxel on glioblastoma xenografts [40]. The other agent tested, the histone deacetylase inhibitor SAHA, only revealed an increase in NIS expression level in one out of four cell lines, and no altered expression of Tg, TPO and TSH-R in any cell line. Others have demonstrated an enhanced NIS expression in thyroid cancer cells as well as a restored TPO, Tg and TSH-R level by SAHA treatment, although higher concentrations of SAHA were used [36,41]. In our study the combination of $1,25(OH)_2D_3$ and paclitaxel/SAHA did not lead to an enhanced redifferentiation profile, which contrasts with Akagi et al. demonstrating an enhanced NIS expression level after co-treatment of $1,25(OH)_2D_3$ and SAHA, but again higher concentrations of SAHA were used [36]. To determine whether $1,25(OH)_2D_3$ potentially qualifies as a clinically relevant inducer of NIS, additional experiments are needed in order to investigate proper NIS function.

As thyroid cancer cell lines result from an *in vitro* evolution of the original tumor cells, they might have lost their susceptibility to anticancer agents. The present clear effects of $1,25(OH)_2D_3$ and analog CD578 in the cell lines used in this study, representing poorly to undifferentiated thyroid cancer, are thus a good argument for further *in vivo* investigation.

In summary, we show potent and promising *in vitro* antiproliferative effects of $1,25(OH)_2D_3$ and especially analog CD578 in combined regimen with paclitaxel and SAHA in anaplastic thyroid cancer cells. These findings warrant further studies to explore the molecular mechanisms and *in vivo* preclinical studies in order to confirm the potent growth inhibitory effects, to explore the mechanisms of interaction between the different anti-cancer agents and

further refine dosing, toxicity and pharmacokinetics in view of evaluation for potential clinical applicability.

Disclosure statement

The authors declare that no competing financial interest exists.

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