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BRIEF REPORT

The pyrazolo[3,4-d]pyrimidine derivative CLM24 has an antineoplastic effect in aggressive dedifferentiated thyroid cancer in vitro

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Abstract

Purpose Compounds with a pyrazolo[3,4-d]pyrimidine nucleus (CLM24, CLM3, and CLM29) with inhibitory activities towards RET protein kinases, EGFR, VEGFR and as antiangiogenic agents had been evaluated in preceding researches in experimental models of thyroid cancer (TC) in vitro and in vivo. Here, we designed a study to evaluate the antineoplastic activity of CLM24 in vitro in primary dedifferentiated thyroid carcinoma (DeDTC) cells and in the AF cell line (a continuous ATC cell line previously obtained from a patient-derived cell culture and immortalized in vitro).

Methods We investigated in vitro CLM24 in human primary cultures from 10 patients with DeDTC and 5 healthy controls, and in the continuous AF cell line (as a comparative reference), evaluating its effect on cell proliferation, apoptosis, migration/invasion, and VEGF-A expression.

Results CLM24 showed a significant antiproliferative effect on the AF cell line (harboring the BRAF p.V600E mutation), along with a marked pro-apoptotic activity. In primary DeDTC cells with/without BRAF p.V600E mutation, CLM24 significantly reduced cell proliferation compared with controls, whereas no effect was observed in primary normal thyrocytes. In DeDTC cells with/without BRAF p.V600E mutation, the proportion of apoptotic cells increased in a dose-dependent manner following CLM24 treatment, which also significantly inhibited cell migration/invasion. Furthermore, CLM24 reduced significantly VEGF-A expression in DeDTC cells, especially at higher concentrations.

Conclusion CLM24 exhibited a potent antineoplastic effect in vitro in primary DeDTC cells, regardless of the presence of the BRAF p.V600E mutation. These encouraging results suggest further studies to assess CLM24 in a possible clinical trial setting.

Keywords dedifferentiated aggressive thyroid cancer · pyrazolo(3,4-d)pyrimidine derivatives · CLM24 · primary cell cultures · tyrosine kinase inhibitors

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Introduction

Among endocrine malignancies, thyroid cancer (TC) is the most frequently diagnosed, with a rising incidence [1]. Differentiated TC (DTC) represent>90% of TCs and are divided into papillary TC (~90%), follicular TC (~10%) and oncocytic carcinoma of the thyroid. Poorly differentiated TCs are ~2–5% of TCs and anaplastic TC (ATC) are <2% [2].

As DTC progresses, tumor cells may become refractory to RAI, and currently available therapeutic strategies (i.e., surgery, chemotherapy, and external beam radiation therapy) can be associated with significant side effects without improving survival [3]. Therefore, there is a need to identify effective systemic therapies for patients with dedifferentiated DTC (DeDTC). Small molecule inhibitors targeting the intracellular tyrosine kinase (TK) linked to different receptors have been approved by US Food and Drug Administration and European Medicines Agency to treat aggressive and refractory TCs [4–7].

In this context, we demonstrated in vitro the antitumoral activity of lenvatinib [8] and vandetanib [9] in human primary ATC (pATC) cells [10], and in vivo in ATC xenotransplants in nude mice. We reported also the in vitro antitumoral activity of pazopanib in pATC cells [11], and of crizotinib in pATC with STRN–ALK fusion [12]. Moreover, we demonstrated the antitumoral and antiangiogenic action of the TK CLM3 in pATCs [13], and CLM24 and CLM29 in the continuous 8305 C cell line and in pATCs [14]. Furthermore, in vitro in pATC CLM94 had an antineoplastic effect and in vivo in CD nu/nu mice [15].

Here, we designed a study to investigate in vitro the antineoplastic effects of CLM24 in primary dedifferentiated aggressive DeDTC cells and in the AF cell line, a continuous ATC cell line previously obtained from a patient-derived cell culture and immortalized in vitro (with BRAF p.V600E mutation), used as a comparative reference, since we have already demonstrated the antineoplastic effects of CLM24 on ATC [14].

Table 1 Clinical data of the patients from whom primary dedifferentiated aggressive thyroid carcinoma (DeDTC) cell cultures were obtained

Number of patients	10
Age	age range, 51–83 (median, 65)
Sex	8 females, 2 males
Tumor size	range, 4–11 cm
Staging according to the Eighth Edition American Joint Committee on Cancer (AJCC) Cancer Staging Manual	Stage II pT3aN1 (2 patients), Stage II pT3b N1 (2 patients), Stage III pT3b N1 (3 patients), Stage III pT4aN1 (3 patients)
K-RAS, N-RAS, H-RAS mutations, RET/PTC1, and RET/PTC3	not detected
BRAF p.V600E mutation	detected in 4/10 patients
TPO, TSH-R, Tg, and NIS	expressed (by immunohistochemistry)

All the patients with DeDTC had been operated again for recurrence of thyroid cancer, not uptaking 131-I. Immunohistochemistry was applied to establish the expression of Tg, sodium/iodide symporter (NIS), TSH receptor, and thyroperoxidase (TPO). Mutations in RET/PTC1, PAX8/PPARγ arrangements, RET/PTC3, K-RAS, BRAF, N-RAS, and H-RAS were evidenced by real-time RT-PCR, both in thyroid DeDTC tissue samples and in human primary DeDTC cell cultures [24, 25]

Materials and methods

Drugs and reagents

CLM24 (N,1-diphenethyl-1 H-pyrazolo[3,4-d]pyrimidin-4-amine) was synthesized by C.L.M. at the Department of Pharmacy of the University of Pisa [14], and stocked in dimethyl sulfoxide (DMSO). The reagents used in this study were sourced from Sigma-Aldrich, Merck (Darmstadt, Germany).

DeDTC samples and their characterization

Thyroid tissue samples were collected from 10 DeDTC patients (Table 1), and from 5 healthy controls (who had undergone parathyroidectomy). The diagnosis of DeDTC was done as before [16]. The enrolled subjects accepted to enter the research, and the approval of this research was granted by the Ethics Committee of the University of Pisa.

AF cell line

The AF cell line is an immortalized ATC cell line (with BRAF p.V600E mutation) established from primary ATC cells that once inoculated sc, could grow in nu/nu mice [15].

Human primary cell cultures

DeDTC cells were prepared as previously reported; the expression of Tg, NIS, TSH receptor, and TPO was established through immunocytochemistry [16]. Normal thyroid follicular cells were also established [14]. Cell cultures were tested at the 4th passage [13–15].

Cell proliferation/viability assay

35,000 cells/mL were plated in 100 µL, and CLM24 (1, 5, 10, 30, and 50 µM) or DMSO (as negative control) were put in quadrupled. After 24 h, the WST-1 test was performed as

recommended by the manufacturer. To detect proliferation, it was used also the cell number counting [16].

Apoptosis

35,000 DeDTC cells/mL were seeded in 100 µL per well, treated for 24 h with CLM24 (1, 5, 10, 30, and 50 µM), and stained with Hoechst 33342 [15]; the apoptosis index was calculated. Furthermore, Annexin binding assay was done, after 24 h of treatments with CLM24 [8].

Migration and invasion assay

Migration and invasion tests were done based on the manufacturer's indications [16]. Migration was conducted for 12 h and invasion for 24 h [15].

VEGF-A

Immunocytochemistry for VEGF was done with anti-VEGF rabbit polyclonal antibodies (Santa Cruz Biotechnology, Dallas, Texas) used at a 1:50 dilution and results were presented as the proportion of positive cells (%) vs. at least 1000 tumoral cells (vs. control, not-treated cells) [11]. Moreover, VEGF-A gene expression was evaluated also by real-time PCR analysis (see Figure legend) [14].

Statistical Analysis

The tests were done in triplicate with DeDTC cells from each patient. The data are reported as mean±standard deviation (SD) or as median and interquartile range for normal variables. Statistical analysis was conducted using STATA, and StatView. One-way ANOVA was performed to correlate the mean group values for normally distributed variables, and the Bonferroni–Dunn test to post hoc comparisons on normally distributed variables.

Results

Primary cell characterization

Thyroidal tissue samples were obtained from 10 DeDTC patients (Table 1), and 5 healthy controls.

The mutations identified in the original tumor samples were retained in the corresponding primary cell cultures.

In vitro studies in the AF cells

CLM24 exhibited a significant inhibition of proliferation in the AF cell line (IC50 28±11 µM) (Fig. 1A), in accordance with cell counting. In the AF cell line the cell number was 19,920±845/100 µL, per well; 19,722±1020 (99%) with CLM24 1 µM; 17,928±1013 (90%) with CLM24 5 µM; 15,737±990 (79%) with CLM24 10 µM; 10,358±1020 (52%) with CLM24 30 µM; 5,578±1040 (28%) with CLM24 50 µM; ($P<0.01$, by ANOVA). Moreover, CLM24 had a significant pro-apoptotic effect on the AF cells (Fig. 1B).

In vitro studies in human primary cells

The WST-1 assay in the 6 DeDTC cells without BRAF p.V600E mutation (Fig. 1C) demonstrated a significant decrease in proliferation vs. control with CLM24 (IC50 35±13 µM; $P<0.01$, by ANOVA). The results on proliferation inhibition by CLM24 in the 4 DeDTC from tumors with BRAF p.V600E (IC50 25±12 µM; $P<0.01$, by ANOVA; Fig. 1E) are comparable to those in absence of BRAF p.V600E. Cell counting confirmed these data (additional data are given in Online Resource 1 for the 6 DeDTC cells without BRAF p.V600E mutation and Online Resource 2 for the 4 DeDTC from tumors with BRAF p.V600E).

WST-1 and cell counting in normal thyroid follicular cells treated with CLM24 reported no significant results vs. control (additional data are given in Online Resource 3).

The % of apoptotic cells in the 6 primary DeDTC without BRAF p.V600E mutation raised in a dose dependent way (Fig. 1D; $P<0.001$, by ANOVA), and this occurred also in the 4 primary DeDTCs with BRAF p.V600E mutation (Fig. 1F; $P<0.001$; by ANOVA). Annexin V confirmed these data (additional data are given in Online Resource 4).

The induction of apoptosis in DeDTC cells with/without BRAF p.V600E mutation by CLM24 was not significantly different ($P>0.05$).

Moreover, CLM24 significantly inhibited ($P<0.001$) the migration of DeDTC cells (Fig. 1Ga). For comparison the proliferation at 12 h was performed, and the inhibition of migration was higher than that of proliferation: expressing control as 100% and treatments as a percentage of it, the proliferation was: 100% with CLM24 1 µM; 91% with CLM24 5 µM; 67% with CLM24 10 µM; 60% with CLM24 30 µM; 40% with CLM24 50 µM. Furthermore, CLM24 significantly inhibited ($P<0.01$) the invasiveness of DeDTC cells (Fig. 1Gb), and such inhibition was higher than that of proliferation at 24 h.

Furthermore, after 72 h, CLM24 significantly reduced VEGF-A expression in DeDTC cells ($P<0.05$; Fig. 1H), and VEGF-A immunostaining. A localized immunoreactivity for VEGF-A was identified in the primary not treated cells which was reduced by CLM24 50 µM (38±9 vs. 26±4; $P<0.05$).

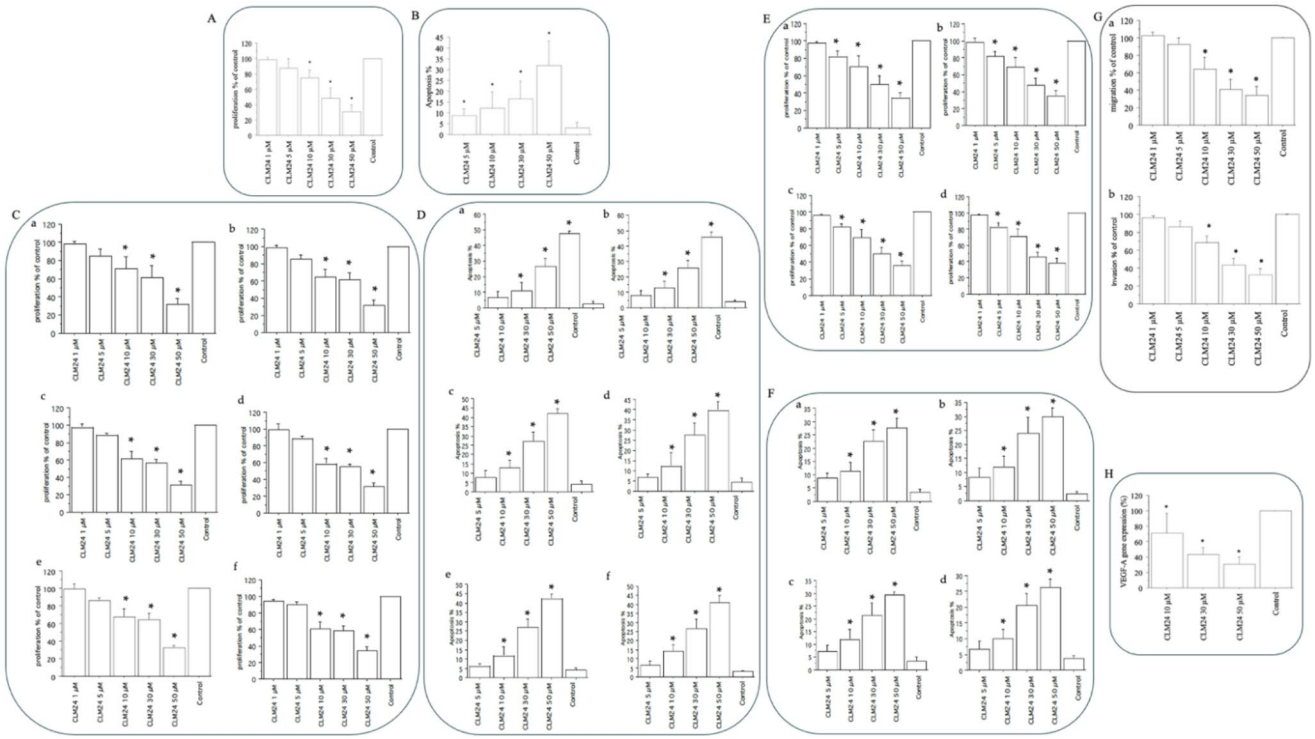

Fig. 1 **A** WST-1 assay (mean ± SD) in AF cells, used for this study as a comparative reference. CLM24 showed dose-dependent antiproliferative activity in AF cells with the BRAF p.V600E mutation after 24 h. Bars are means ± SD (* $P<0.05$ or less vs. control expressed as 100% of growth; Bonferroni–Dunn test). **B** CLM24 had a concentration-dependent pro-apoptotic action on the AF cells. Bars are means ± SD (* $P<0.05$ or less vs. control; Bonferroni–Dunn test). **C** WST-1 assay in the 6 primary DeDTC cells without BRAF p.V600E mutation treated for 24 h with CLM24. A significant decrease in proliferation vs. control was demonstrated. Bars are means ± SD (* $P<0.05$ or less vs. control; Bonferroni-Dunn test). **D** Apoptosis in the 6 primary DeDTC cells without BRAF p.V600E mutation. The apoptosis index was obtained through Hoechst (see "Methods"). Data are expressed as mean ± SD (* $P<0.001$ vs. control; Bonferroni–Dunn test). **E** WST-1 assay in the 4 primary DeDTC cells with BRAF p.V600E mutation treated for 24 h with CLM24. A significant decrease in proliferation vs. control was demonstrated. Bars are means ± SD (* $P<0.05$ or less vs. control; Bonferroni-Dunn test). **F** Apoptosis in the 4 primary DeDTC cells with BRAF p.V600E mutation. The apoptosis index was obtained through Hoechst (see "Methods"). Data are expressed as mean ± SD (* $P<0.001$ vs. control; Bonferroni–Dunn test). **G** Migration (**a**) and invasion (**b**) in primary DeDTC cells treated with increasing concentrations of CLM24. CLM24 10, 30 50 μM inhibited migration and invasion. The results are the mean of the 10 samples. Bars are means ± SD (* $P<0.05$ for CLM24 vs. 10% fetal calf serum (= control); Bonferroni–Dunn test). **H** VEGF-A gene expression in DeDTC cells treated with CLM24 (50, 30 and 10 μM) for 72 h (mean ± SD of all samples). CLM24 significantly reduced VEGF-A expression, especially at the higher concentrations. The results are the mean of the 10 samples. Bars are the mean ± SD (* $P<0.05$ vs. control; Bonferroni–Dunn test). cDNA was generated by reverse transcription of RNA and subsequently amplified by quantitative real-time PCR with the Applied Biosystems 7900HT system. The VEGF-A primer was purchased from Applied Biosystems (Thermo Fisher Scientific). Amplifications were normalized to glyceraldehyde 3-phosphate dehydrogenase, and the quantitation of gene expression was performed using ΔΔCt calculation; the amount of target was given as $2^{-\Delta\Delta Ct}$. Through the panels 1 A-1 H, "Control" means not treated cells

Discussion

The pyrazolo[3,4-d]pyrimidine derivative CLM24, already studied as a TK inhibitor, reduced the growth of the AF cell line and primary DeDTC cells in vitro, and increased the apoptosis, regardless of the presence of the BRAF mutation. CLM24 also inhibited the migration and invasiveness of primary DeDTC cells, demonstrating a potent antineoplastic effect in vitro.

To date, other pyrazolo[3,4-d]pyrimidines, such as PP1 [17], PP2 [18] and Si34 [19] have been studied in TC. The pyrazolopyrimidine PP1 has been previously reported to exert a strong inhibitory effect on RET TK [20].

The pyrazolopyrimidine PP2 decreased the MAP kinase signaling, and it inhibited the proliferation and invasive phenotype of human TC cells with RET/PTC1 [18] rearrangements and also c-Src and related kinases [21].

This is true also for Si34 [18], that had inhibitory effects on the TT and MZ-CRC-1 tumor cell lines (human medullary TC derived cells), via the inhibition of the c-Src TK.

Noteworthy, in the primary DeDTC cells we investigated, the observed anti-proliferative action of CLM24 was not dependent from the presence/absence of the BRAF mutation. These data agree with the idea that CLM24 is suggested for multiple inhibition of signal transduction, involving the TKs RET, EGFR, VEGFR, and has an antiangiogenic effect. The

functional efficacy of small molecule kinase inhibitors can be bypassed by compensatory activation of other kinases, and an advantage of multikinase inhibitors is their ability to target multiple kinases simultaneously, thereby overcoming this resistance mechanism [21].

The antineoplastic action of CLM24 could derive from its antiproliferative effect combined with an increased apoptosis in tumor cells, and also from the inhibition of neoplastic neovascularization and of ERK1/2 phosphorylation. This has been demonstrated in vivo for other pyrazole-pyrimidine derivatives [9], and in this study CLM24 reduced significantly VEGF-A expression in DeDTC cells, especially at higher concentrations. In the last years the emergence of VEGF-A-targeted therapy alone or in rational combinations has revolutionized the treatment of different types of cancer [22].

To our knowledge, this is the first study that evaluated the antineoplastic activity of CLM24 in primary human DeDTC cells. Till nowadays, continuous thyroid tumoral cells have been considered as preclinical models, but they rearrange themselves to the in vitro environment, losing certain specific features involved in normal thyroidal function.

The in vitro screening of targeted drugs conducted in human primary cancer cells established from a single patient can suggest a 60% positive predictive value of clinical response [23], and an in vivo non-responsivity (with a 90% negative predictive value). The possibility of investigating the sensitivity of each subject's primary DeDTC cells to different TKIs could avoid the administration of ineffective drugs.

The major limitation of the present study is the lack of in vivo experiments, which are needed to corroborate these results, paving the way for future clinical studies in patients with DeDTC.

To conclude, the strong antitumor action of the pyrazolo[3,4-d]pyrimidine derivative CLM24 was demonstrated in BRAF p.V600E mutated AF cells and human primary aggressive DeDTC cells. The highly promising results obtained suggest that CLM24 is a derivative deserving further investigation for potential clinical efficacy.

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Data availability The data presented in this study are available within the article.

Declarations

Informed consent Informed consent was obtained from all subjects involved in the study.

Conflict of interests The authors declare no competing interests.

Institutional Review Board Statement The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee for Clinical trials of Pisa University Hospital (date of approval 18 November 2012).

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