Expression of Tissue Transglutaminase in Human Thyroid Cancer Cell Lines: Effect of Novel Enantiopure Triazole Derivatives

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Abstract

A synthesis of (-)5b and (-)6d according to enzymatic resolution of racemic triazole derivatives 5b and 6d was performed with Lipzyme in 1,4-dioxane, using vinyl benzoate as the acyl donor. The effect of the compounds on TG2 overexpression in human thyroid cancer cell lines cultures at two degrees of malignancy and invasiveness was assessed. The involvement of TG2 in the activation of the apoptotic pathway is also shown. We found that the obtained compounds are able to reduce TG2 overexpression in follicular (FTC-133) and anaplastic (8305C) human thyroid cancer cell lines either in the cytosol, or in the nuclear compartment, activating also the apoptotic pathway. Our data suggest that they may represent new anticancer agents for human thyroid cancer.

Keywords: Modified nucleosides; Thyroid cancers; Isoxazolidines; Triazole; Antitumor agents; Tissue transglutaminase

Introduction

TG2 is the ubiquitous and the most abundantly expressed member of the TGs family [1]. It catalyses the formation of protein cross-links, namely Nε-(γ-glutamyl) lysine bridges, between the side chains of peptide-bound Glutamine and Lysine residues [2]. TG2 is a unique member of the TGs family, in that it can also hydrolyse ATP and GTP; when bound to GTP, it functions as a member of the family of GTP-binding proteins [3-6]. TG2 mediates reactions associated with long-term potentiation, synapse formation, tissue differentiation, cell survival [7], apoptosis [8,9], cell adhesion, protein disulfide isomerase [10], kinase [11] and scaffold activities [12]. It is present in the cell, including the extracellular matrix, plasma membrane, cytosol, mitochondria [13] and nucleus [14,15]. The multiple functions of the protein depends on its intracellular localization [16]. In particular, when it is localized in the cytosol differentially controls apoptosis in a stimuli-dependent manner, and its transamidating activity is essential for its pro-apoptotic effects [17,18]. In contrast, when TG2 is localized into the nuclear compartment, it phosphorylates different proteins, including retinoblastoma protein (Rb), a substrate for TG2 kinase activity [19]. Aberrant TG2 expression contributes to a variety of disease processes [20], including cancer [21-25], neurodegenerative diseases [26-31], autoimmune diseases, such as celiac disease [32,33], rheumatoid arthritis [34,35], tissue fibrosis [36].

It has been shown that overexpression of TG2 can lead to an increase of apoptosis, while a decrease in the levels of TG2 involves a decrease both spontaneous apoptosis and inhibits caspase-3 [37,38].

TG2 inhibits its ability to catalyze reactions of protein cross-linking and restores the activation of caspase-3 [39]. Paradoxically, the expression of TG2 can also protect cells from apoptosis [39]. Although some studies have indicated that TG2 may suppress tumor growth and improve the growth inhibitory effects of anti-cancer agents, several studies have presented both roles of survival and anti-apoptotic of TG2 in malignant cells [40].

TG2 represents a single target that can modulate multiple pathways and functions in cancer cells and thus its inhibition can simultaneously deprive cancer cells of multiple pathways that are critical for their growth and survival [41]. Many inhibitors of TG2, including triazole derivatives have been examined [42-48], and a relationship with the development of various types of tumors [49,50], including thyroid cancer has been found. In particular, 1,2,3-triazole motif is exceedingly stable to basic or acidic hydrolysis and interacts strongly with biological targets through hydrogen bonding to nitrogen atoms as well as through dipole-dipole and π-stacking interactions [51].

On these basis, we have, recently, synthesized a series of 3-hydroxymethyl-5- (1H-1,2,3-triazol) isoxazolidines 5a-f and 6a-f, belonging to the class of N,O-modified nucleosides [52-59], according to a synthetic approach based on the cycloaddition reaction, under microwave irradiation (Mw), of nitronone with 4-substituted 1-vinyl triazoles 2a-f (Scheme 1).

Preliminary biological studies have shown that compounds 5b and 6d were able to activate the apoptotic pathway in follicular (FTC-133) and anaplastic (8305C) human thyroid cancer cell lines, as...
representative of two aggressive types of human thyroid cancer, poorly differentiated and dedifferentiated, respectively [60].

Herein, we report the enzymatic resolution of triazole derivatives of (-)-5b and (-)-6d and the effect of (-)-5b and (-)-6d on TG2 overexpression in follicular and anaplastic human thyroid cancer cell lines [61,62]. Furthermore, the involvement of TG2 in the activation of the apoptotic pathway is shown.

Experimental Section

Chemical synthesis

Solvents and reagents were used as received from commercial sources. Thin layer chromatographic separations were performed on Merck silica gel 60-1024 pre-coated aluminum plates (Merck, Darmstadt, Germany). Flash chromatography was accomplished on Merck silica gel (200–400 mesh). Preparative separations were carried out by a Buchi C-601MP LC (Buchi Italia S.r.l., Milano, Italy), using Merck silica gel 0.040-0.063 mm and the eluting solvents were delivered by a pump at the low flow rate of 3.0-7.0 mL/min. 1H-NMR (500 MHz) and 13C-NMR (125 MHz) spectra were recorded in CDCl3, on a Varian 500 instrument (Agilent Technologies, Palo Alto, Ca, USA).

M. mielei (Lipoyzime) was purchased from Aldrich. Optical rotations were recorded on a DIP 135 JASCO instrument using a φ 5.5 x 100 mm cell. The enantiomeric excesses and substrate conversions were determined by chiral HPLC analysis using a Chiralcel OD (Daicel Chemical Industries) column. C-[tert-butylidiphenylsilyl]oxy)-N-methyl nitronel, vinyl triazoles 2b,d, and racemic 5b and 6d were prepared according to the methods described in literature [53].

General procedure for the synthesis of (-)-5b and (-)-6d: To a solution of racemic substrates 5b and 6d (1 mmol) in 1,4-dioxane (150 mL) was added 2.5 g of M Lipoyzime and vinyl benzoate (1 mmol). The resulting mixture was incubated at 45°C and shaken (300 rpm) until 50% of substrate conversion detected by HPLC analysis. The reaction was stopped and filtered by Celite filter. The resulting solution was evaporated under vacuum, and the residue was purified by MPLC (CH2Cl2/MeOH, 98:2) to afford enantiopure (-)-5b and (-)-6d in 40 and 45% yield respectively.

(3RS,5RS)-2-methyl-5-(4-propyl-1H-1,2,3-triazol-1-yl)isoxazolidin-3-yl)methanol(-)-5b: Yellow oil: 1H NMR (500 MHz, CDCl3) δ: 0.94 (3H, t, J=7.1 Hz), 1.58-1.73 (2H, m), 2.65 (2H, t, J=7.5 Hz), 2.80 (3H, s), 2.81-2.96 (2H, m), 2.97-3.06 (1H, m), 3.75 (2H, ddd, J=13.4, 11.7, 3.6 Hz), 6.26 (1H, dd, J=7.8, 2.2 Hz), 7.71 (1H, s). 13C NMR (126 MHz, CDCl3) δ: 13.9, 22.7, 27.7, 39.0, 44.4, 61.1, 69.0, 85.8, 119.6, 148.5. [α]25 : D=-11.2 (c 0.8 CHCl3). Anal. calcd for C11H16N4O2: C, 53.08; H, 8.02; N, 25.89; found C, 52.89; H, 7.96; N, 24.86.

(3RS,5SR)-2-methyl-5-(4-pentyphenyl)-1H-1,2,3-triazol-1-yl)isoxazolidin-3-yl)methanol(-)-6d: White solid, mp: 99-100°C. 1H NMR (500 MHz, CDCl3) δ: 0.89 (3H, t, J=6.9 Hz), 1.26-1.40 (4H, m), 1.60-1.66 (2H, m), 2.53 (2H, t, J=7.4 Hz), 2.76 (3H, s), 2.80-2.93 (2H, m), 3.48-3.58 (1H, m), 3.70 (2H, ddd, J=16.5, 11.5, 4.2 Hz), 6.12-6.21 (1H, m), 7.24 (2H, d, J=8.0 Hz), 7.74 (2H, d, J=8.0 Hz), 7.94 (1H, s). 13C NMR (126 MHz, CDCl3) δ: 14.1, 22.7, 31.1, 31.6, 35.8, 36.8, 46.7, 62.1, 68.0, 88.1, 119.2, 125.8, 127.7, 129.1, 143.5, 148.6. [α]25 : D=+0.96 (c 0.4 CHCl3). Anal. calcd for C26H26N4O2: C, 65.43; H, 7.93; N, 16.96; found C, 65.41; H, 7.90; N, 16.89.

The NMR spectra and elemental analysis were identical with the literature data [53].

Cells and reagents

Human thyroid tumor cell lines: FTC-133 and 8305C, were obtained from Interlab Cell Line Collection (ICLC). Dulbecco’s Modified Eagle Medium (DMEM) and Minimum essential Medium (MEM) containing 2 mM Gluta-MAX (GIBCO), Ham’s F12 (GIBCO), non-essential amino acids, heat inactivated- Foetal Bovine Serum (FBS, GIBCO), Normal Goat Serum (NGS, GIBCO), Streptomycin and penicillin antibiotics, Trypsin-EDTA 0.05% solution, and Mouse anti-Human Transferrin Receptor (TIR-1) monoclonal antibody were obtained from Invitrogen (Milano, Italia). Lab-Tek® Chamber Slides II, 3(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide salts (MTT), and other chemicals (DMSO, Tris-HCl, NaCl, PMSF and Tween 20) of analytical grade were obtained from Sigma-Aldrich (Milano, Italy) and microplate spectrophotometer reader (Titertek Multiskan; Flow Laboratories, Helsinki, Finland) to measure the optical density of each well. 3-hydroxymethyl-5- (1H-1,2,3-triazol) isoxazolines 5b and 6d [53]. Mouse monoclonal antibody against TG2 was from NeoMarkers (Biopitca, Milano, Italy). Mouse monoclonal antibody against caspase-3 was from Becton Dickinson (Milan, Italy). Mouse monoclonal antibody against β-tubulin and tetrarhadamine isothiocyanate (TRITC)-conjugated anti-mouse IgG were from Chemicon (Prodotti Gianni, Milano, Italy). ApoAlert DNA fragmentation assay kit was from Clontech (Super Signal West Pico Chemiluminescent Substrate was from Thermo Scientific (Milan, Italy).

Cell cultures

FTC-133 and 8305C cell lines were suspended in appropriate medium and plated in flasks at a final density of 2 x 10^4 cells to 10^5 cells/ well. Specifically the medium for FTC-133 cell lines was: DMEM containing 2 mM Gluta-MAX, 10% FBS, streptomycin (50 µg/ml), penicillin (50 U/ml), whereas, the medium for 8305C cell lines was: MEM containing 2 mM Gluta-MAX, 10% FBS, streptomycin (50 µg/ml), penicillin (50 U/ml), and 1% Non-Essential Amino Acids. Cell lines were then incubated at 37°C in humidified atmosphere containing 5% CO2 and the medium was replaced every 2 or 3 days. When the cultures were about 85-90% confluent, cells were trypsinized by 0.05% trypsin and 0.53 mM EDTA at 37°C in humidified atmosphere containing 5% CO2 for 5 min. Trypsination was stopped by adding 20% FBS, resuspended and plated in flasks fed with fresh basic complete media. Cells were seeded again at 1:4 density ratio and incubated at 37°C in humidified atmosphere containing 5% CO2.

Treatment of cell cultures

FTC-133 and 8305C cell line cultures were replicated on to Lab-Tek Chamber Slides II at a final density of 1 x 10^4 cells/well, or in 75 cm²flasks at a final density of 1 x 10^5 cells/mL and fed in fresh complete medium. In preliminary experiments, all of the cell cultures were exposed to different concentrations of synthesized triazole derivatives (-)-5b and (-)-6d [53] (0.5, 1, 5, 10, 25, 50, 100 µM) for 12, 24, 48 hr, in order to establish the optimal concentrations and their exposure times to the triazole derivate [28,62,63]. For this purpose, MTT test and morphological characterization were utilized [30].

MTT bioassay

Cell survival analysis was performed by MTT reduction assay, evaluating mitochondrial dehydrogenase activity [28,30]. The test was conducted according to the method described by Mosmann (1983). Cells were set up 6 x 10^4 cells per well of a 96-multiwell, flat-bottomed,
200 µl microplate, and maintained at 37°C in a humidified 5% CO2/95% air mixture. At the end of treatment time 20µL of 0.5% DMSO in (pH 7.4) PBS were added to each microwell. After 1 hr of incubation with the reagent, the supernatant was removed and replaced with 200 µL of dimethyl sulfoxide (DMSO).

The optical density of each well was measured with a microplate spectrophotometer reader (Titertek Multiskan; Flow Laboratories, Helsinki, Finland) at λ 570 nm. Since all the compounds were dissolved in DMSO, some cultures were treated with the same concentration of DMSO. No significant changes in cellular viability in DMSO-treated FTC-133 and 8305C cancer cell line cultures was found, when compared with the respective untreated control. Thus, the effect of drug- treatment of cancer cell line cultures was compared with that DMSO-treated alone, and it was used as control.

Immunocytochemistry

Expression and localization of TG2 and caspase-3 in untreated and 5 µM triazole derivatives treated FTC-133 and 8305C culture cell lines was identified by immunocytochemical procedures [14,28,30,31].

The cells were fixed by exposing to 4% paraformaldehyde in 0.1 M PBS for 30 min. The cells were successively incubated overnight in the primary antibodies: anti-TG2 monoclonal antibody (1:500) or mouse anti-caspase-3 monoclonal antibody (1:500). Then, coverslips were incubated with a secondary antibodies, TRITC-conjugated anti-mouse IgG anti-mouse antibody (1:200). Coverslips were washed, mounted in PBS/glycerol (50:50), placed on glass microscope slides and analysed on a fluorescent microscopy (Leica, Germany). No non-specific staining of FTC-133 and 8305C was observed in control incubations in which the primary antibody was omitted.

TUNEL test

The ApoAlert DNA fragmentation assay kit detecting nuclear DNA fragmentation kit, a hallmark of apoptosis, was used. The ApoAlert DNA fragmentation assay incorporates fluorescein-dUTP at the free 3’-hydroxyl ends of the fragmented DNA using TUNEL and was performed according to the user’s manual. FTC-133 and 8305C cell cultures, untreated and treated for 24 h with 5 µM of Gemcitabine, or (-)5b or (-)6d compounds were made up according to the user’s manual. Cells were mounted and visualized directly by fluorescence microscopy (Leica, Germany) with either a propidium iodide (PI) filter alone or a FITC filter alone. According to the user’s manual, apoptotic cells appear green with the FITC filter alone while non apoptotic cells appear red under the dual- pass FITC/PI filter set. We focused on 10 random microscopic fields for each dish. In each microscopic field we counted the number of apoptotic cells and we compared this number with the respective untreated control. Thus, the effect of drug- treatment of cancer cell line cultures was compared with that DMSO-treated alone, and it was used as control.

Statistical analysis

Data were statistically analysed using one-way analysis of variance (one-way ANOVA) followed by post hoc Holm-Sidak test to estimate significant differences among groups. Data were reported as mean of (one-way ANOVA) followed by post hoc Holm-Sidak test to estimate

Results and Discussion

Chemistry

The racemic triazoles (+/-) 5b and (+/-)6d, obtained by 1,3-dipolar cycloaddition reaction of C-[(tert-butyldiphenylsilyl)oxy]-N-methyl nitroene 1 with vinyl triazoles 2b,d, followed by TBAF treatment, were converted into the enantiomeric pure (-)5b and (-)6d by an esterification reaction, catalyzed by Lipoyzyme 1M in 1,4-dioxane, using vinyl benzoate as the acyl donor (Scheme 2).

The esterification reaction was monitored by HPLC chiral analysis and stopped after 50% of conversion. The reaction mixture was chromatographed on silica gel eluting with CH3Cl/Methanol 98:2 to afford the benzoate derivatives 7b and 7d as mixture of enantiomers, and (-)5b, and (-)6d in enantiomeric pure forms. The absolute configuration reported as (3S,5R)- for 5b and (3R,5S)- for 6d, see experimental section, is arbitrary and can be inverted. The benzoate derivatives 7b and 7d were then, dissolved in 1,4-dioxane and the alcoholysis using n-butanol in the presence of Lipoyzyme 1 M was attempted. Unfortunately, this reaction occurred very slowly giving a negligible amount of (+)5b and (+)6d; thus further efforts to obtain these enantiomers in good yields were abandoned.

Cytotoxic effect of (-)5b and (-)6d

In preliminary experiments, some cultures of both cell lines were treated with different concentrations of (-)5b and (-)6d (1, 5, 10, 20, 40, 80 and 100 µM) for 24 hr, to perform the MTT test. It was found that the compounds were able to reduce the cell viability at concentration of 5 µM for 24 hr. The cytotoxic effect appeared more evident in FTC-133 cell lines (Figure 1).

The 50% cytotoxic inhibitory concentration (IC50), causing 50% decreasing in cell proliferations, obtained graphically from dose-effect curves using Prism 5.0 (GraphPad Software Inc.). In particular, compound (-)5b has shown an IC50 for FTC-133=4.1 µM and=7.6 µM for 8305C, while compound (-)6d has shown an IC50 for FTC-133=3.9 µM and=7.9 µM for 8305C. The effect was compared with Gemcitabine.
Effect of (-)5b and (-)6d on TG2

Figures 2 and 3 show the expression of TG2 in FTC-133 human thyroid cancer cell lines untreated or treated with 5 µM (-)5b, (-)6d triazole derivatives and Gemcitabine for 24 hr evaluated by immunocytochemical techniques on single cell and by Western Blot analysis on total cell lysates. Immunocytochemical analysis for the positivity of untreated-cells, used as a control, TG2 showed a high levels of the protein in the both cell lines. The protein appeared prevalently localized in the both cell lines the cytosol and also in the nuclear compartment. The treatment of the cells with (-)5b and (-)6d or Gemcitabine induced a dramatic reduction of the protein levels. The quantification and statistical analysis of TG2 immunolabeling obtained and collected from 4 fields/cover slip of four separate experiments. No non-specific staining of cells was observed in control incubations in which the primary antibody was omitted.

TG2 into the nuclear compartment, which is related to the increase of intracellular Ca^{2+} concentration. Nuclear TG2 plays an important role in the regulation gene expression via post-translational modification of (or interaction with) transcriptional factors and related proteins [11,19].

Through this mechanism, TG2 controls cell growth or survival, differentiation and apoptosis, and is involved in the pathogenesis and/or treatment of cancers. The balance between import from the cytoplasm to the nucleus, and export from the nucleus to the cytoplasm, determines the level of TG2 in the nucleus [31].

Figures 4 and 5 report a representative immunoblot and densitometric analysis of TG2 expression in FTC-133 and 8305C performed by Western blotting experiments on total cellular lysates.

used as reference compound (IC_{50}; FTC-133=3.2 µM and 8305C=4.8 µM) [64].
An overexpression of TG2 was observed untreated FTC-133 and 8305C human thyroid cancer cell lines. The treatment of the cells with (-)5b and (-)6d or Gemcitabine induced a dramatic reduction of the protein levels. FTC-133 cells were more susceptible to treatment with the synthesized derivatives, than the 8305C cell lines.

Effect of (-)5b and (-)6d on DNA fragmentation in thyroid cancer cell lines

To assess if the triazole compounds were able to activate the apoptotic pathway and involvement of TG2 in its activation, we evaluated caspase-3 cleavage and DNA fragmentation by TUNEL.
test. Figures 6 and 7 show caspase-3 cleavage performed by immunocytochemical procedures in FTC-133 and 8305C cancer cell lines in absence or in presence of Gemcitabine or (-)5b or (-)-6d. A significant number of positive cells to caspase-3 in the both cell lines exposed to drugs was found. The percentage of positive cells was higher in cell cultures of follicular thyroid cancer, when compared with the anaplastic cancer cell lines.

The ability of (-)5b or (-)-6d compounds to activate the apoptotic pathway by measuring DNA fragmentation by TUNEL test (terminal deoxynucleotidyl-transferase mediated dUTP nick-end- labeling test) was also assessed. The effect was also compared with Gemcitabine. This set of experiments showed a significant increase of DNA fragmentation in FTC-133 and 8305C cells treated with all the compounds, when compared with the respective controls. The effect appeared more evident in FTC-133 cancer cell lines (Figures 8 and 9). The obtained data show that the triazole derivatives induced DNA fragmentation depending on the degree of tumor invasiveness.

Conclusions

In summary, we report an efficient synthesis of (-)5b and (-)-6d according to enzymatic resolution of racemic triazole derivatives 5b and 6d performed with Lipozyme 1M in 1,4-dioxane, using vinyl benzoate as the acyl donor. Biological tests indicate that the obtained compounds are able to reduce TG2 overexpression in follicular and anaplastic human thyroid cancer cell lines either in the cytosol, or in
the nuclear compartment, activating also the apoptotic pathway and suggest that they may represent new potential anticancer agents.

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References


