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Full Length Research Paper

Synthesis and antiproliferative activity of diethyl 5-acetyl-4-methyl- 6-(2-fluorophenylimino)-6H-thiopyran-2,3-dicarboxylate (3TM)

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Diethyl 5-acetyl-4-methyl-6-(2-fluorophenylimino)-6H-thiopyran-2,3-dicarboxylate (3TM) was synthesized and the antiproliferative activity of 3TM is reported here. Compound 3TM inhibits the growth of human colon cancer HCT-15 with an IC_{50} value of 4.5 µM and breast cancer MCF-7 with an IC_{50} value of 7 µM in a dose/time dependent manner by using sulforhodamine B assay. Moreover, suppression of clonogenic activity occurs after exposure to 3TM at a concentration of 3 µM for HCT-15 and of 5 µM for MCF-7. The effect of ligand complexation on DNA structure led to the overall affinity constant of $K_{3M\text{-}DNA} = 2.4 \times 10^4$ M^{-1}.

Key words: Diethyl 5-acetyl-4-methyl-6-(2-fluorophenylimino)-6H-thiopyran-2,3-dicarboxylate (3TM), synthesis, antiproliferative activity.

INTRODUCTION

The search for new biologically active agents is an important task for medicinal chemists, especially for investigators who are attempting to develop antitumor agents. In this regards, heterocyclic compounds have varying utility and interesting chemistry and are important due to their role in biological and chemical systems in various ways. Thiopyrans are used as key units in bioorganic chemistry and as versatile building blocks in organic synthesis (Vedejs and Krafft, 1982; Hollick et al., 2003; Yoshimura et al., 2009) and has been reported to have various pharmaceutical activities such as anti-bacteria (Brown et al., 2002), anti-hyperplasia (Quaglia et al., 2002), anti-psychiatric (Van Vliet et al., 2000), and anticancer activities (Wang et al., 2006; Sugita et al., 2001). In view of their wide range of biological activities, we decided to synthesize diethyl 5-acetyl-4-methyl-6-(2-fluoro-phenylimino)-6H-thiopyran-2,3-dicarboxylate (3TM) and study in detail the dose/time-dependent antipro-liferative activity and the anticlonogenic properties of 3TM on two cancer cell lines, MCF-7 (breast cancer) and HCT-15 (colon cancer). There is evidence that anticancer activity was due to the interaction between the drug and the base pairs of DNA and interference with normal functioning of the enzyme topoisomerase II that was involved in the breaking and releasing of DNA strands (Kidwai et al., 2006). In our previous works, we reported synthesis and biological evaluation of different heterocyclic compounds and their DNA binding interaction studies (Nafisi et al., 2008; Rajabi et al., 2010; Mehrzad et al., 2010; Bertini et al., 2010). Therefore, we decided to study the effect of ligand complexation on DNA structure and overall affinity constant calculated by UV-Vis spectroscopy.

MATERIALS AND METHODS

Trypsin, trypan blue, antibiotic and antimycotic agent, fetal bovine serum (FBS), sulforhodamine B (SRB), and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. Chemicals and solvents for DPTM synthesis were purchased from Sigma-Aldrich Italia. NMR spectra were obtained with a Varian Gemini 200MHz spectrometer. Chemical shifts (*d*) are reported in parts per million downfield from tetramethylsilane using solvent references. Electron impact (EI, 70 eV) mass spectra were obtained on a ThermoQuest

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Finnigan GCQ Plus mass spectrometer. Chromatographic separations were performed on silica gel columns by flash (Kieselgel 40, 0.04 – 0.06 mm; Merck) or gravity column (Kieselgel 60, 0.063–0.200 mm; Merck) chromatography. Reactions were followed by thin layer chromatography on Merck aluminum silica gel (60 F254) sheets that were visualized under a UV lamp. Evaporation was performed *in vacuo* (rotating evaporator).

Chemical synthesis of 3TM

To a stirred solution of dimethyl acetylenedicarboxylate, 2 (2 mmol) and arylisothiocyanate 1, (2 mmol) in 10 ml of acetonitrile was added to a mixture of 1,3-dicarbonyl (2 mmol) and sodium hydride (2 mmol) in acetonitrile at room temperature. The reaction mixture was stirred for 8 h. The solvent was removed under reduced pressure and the residue was separated by silica gel column chromatography (Merck 230 - 400 mesh) using *n*-hexane-EtOAc (5:1) as eluent to give 3TM. Oil, yield: 0.61 g (85%). IR (KBr) (ν_{max}/cm^{-1}): 1729 (C=O), 1719 (C=O), 1643 (C=O), 1540, 1435, 1327, 1210, 1100 cm^{-1}; ^{1}H-NMR (500MHz, $CDCl_3$): δ = 1.27 (3 H, t, ^3J = 7.2 Hz, Me), 1.37 (3 H, t, ^3J = 7.2 Hz, Me), 2.05 (3 H, s, Me), 2.52 (3 H, s, Me), 4.25 (2 H, q, ^3J = 7.2 Hz, OCH_2), 4.37 (2 H, q, ^3J = 7.2 Hz, OCH_2), 6.86-7.13 (4 H, m, 4 CH); ^{13}C-NMR (125.7MHz, $CDCl_3$): δ = 13.9 (Me), 14.0 (Me), 17.4 (Me), 30.7 (Me), 62.4 (OCH_2), 63.2 (OCH_2), 116.7 (d, $^2J_{CF}$ = 19.5 Hz, CH), 125.0 (d, $^4J_{CF}$ = 3.2 Hz, CH), 126.0 (d, $^3J_{CF}$ = 7.5 Hz, CH), 129.0 (C), 130.4 (d, $^3J_{CF}$ = 7.3 Hz, CH), 132.4 (C), 134.0 (C), 135.8 (C), 136.5 (d, $^2J_{CF}$ = 72.4 Hz, C-N), 151.2 (d, $^1J_{CF}$ = 285.4 Hz, C), 155.8 (C=N), 160.7 (C=O), 166.3 (C=O), 202.7 (C=O); Anal. Calcd for $C_{20}H_{20}FNO_5S$: C, 59.25; H, 4.97; N, 3.45 found: C, 59.14; H, 4.75; N, 3.27. Oil, yield: 0.73 g (90%).

Cell culture and cell morphology

Human breast cancer MCF-7 and colon cancer HCT-15 cell lines were supplied from ATCC and grown as a monolayer in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 2 mM glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin. Cultures were maintained at 37°C with 5% CO_2 in a humidified atmosphere. Cell shape and morphology of treated and untreated cells were viewed using an inverted phase-contrast microscope model Zeiss and photographed using a Nikon camera attached to the microscope.

***In vitro* evaluation of cytotoxic activity**

Growth activity of 3TM *in vitro* was evaluated by the SRB assay. 3TM stock solution (10 mM in DMSO) was stored at 4°C and diluted with Dulbecco's modified Eagle's medium to 0.1 to 1 µM at room temperature before treatment. The final percentage of DMSO in the reaction mixture was <1% (v/v). Cells (2 × 10^3 cells/well) were plated in 96-wells plates and incubated in medium for 24 h. Serial dilutions of individual compounds were added. The plates were incubated at 37°C for 72 h before addition of 3TM. The assay was terminated by the addition of 50 µl of ice-cold trichloroacetic acid (final concentration, 10% TCA) and incubated for 60 min at 4°C. The plates were washed five times with distilled water and air-dried. SRB solution (50 µl) at 0.4% (w/v) in 1% acetic acid was added to each of the wells, and plates were incubated for 30 min at room temperature. The residual dye was removed by washing five times with 1% acetic acid. The plates were air-dried or under hood. Bound stain was subsequently eluted with 10 mM Trizma base, and the absorbance was read on an ELISA plate reader at a wavelength of 540 nm and used as a relative measure of viable cell number. The percentage of growth inhibition was calculated by using the equation: percentage growth inhibition (1-A_t/A_c) x 100, where A_t and A_c represent the absorbance in treated and control cultures, respectively. IC_{50} was determined by interpolation from dose-response curves.

Clonogenic assay

MCF-7 and HCT-15 cells were plated at a density of 700 cells/well in 12-well plates and treated with 0, 5, 10, 25, 50, 80 and 100 µM 3TM for 14 days. Plates were rinsed in PBS; colonies were methanol-fixed and stained with 10% Giemsa; and clones were counted under a light microscope.

DNA titration experiments

The absorbance at 260 and 280 nm was recorded, in order to check the protein content of DNA solution. DNA (5 mg/ml) was dissolved in distilled water (pH 7) at 4°C for 24 h with occasional stirring to ensure the formation of a homogeneous solution. The final concentration of the DNA solution was determined spectrophotometrically at 260 nm using molar extinction coefficient ε_{260} = 6600 cm^{-1} M^{-1} (expressed as molarity of phosphate groups). The UV absorbance at 260 nm of a diluted solution (1/187.5) of DNA used in our experiments was 0.666 and the final concentration of the DNA solution was 12.5 mM in DNA. The appropriate amounts of 3TM (0.05 to 12.5 mM) were prepared in distilled water and added dropwise to DNA solution in order to attain the desired ligand/DNA molar ratios (r) of 1/40, 1/20, 1/10, 1/5, 1/2 and 1 with a final DNA concentration of 6.25 mM. The pH of the solutions was adjusted at 7.0 ± 0.2 using NaOH solution.

RESULTS AND DISCUSSION

Chemical synthesis of 3TM

Synthesis of diethyl 5-acetyl-4-methyl- 6-(2-fluoro-phenylimino)-*6H*-thiopyran-2,3-dicarboxylate is described via reaction between 1,3-dicarbonyls, electron deficient acetylenic compounds such as dimethyl acetylenedi-carboxylate (DMAD) and aryleisothiocyanate in the presence of sodium hydride as a base (Figure 1) in acetonitrile at room temperature reaction mixture which was stirred for 8 h. The solvent was removed under reduced pressure and the residue was separated by silica gel column chromatography to give 3TM.

Cytotoxic evaluation *in vitro*

The compound 3TM was evaluated for its growth inhibitory effect on HCT-15 colon and MCF-7 breast cancer cell lines. The cells were treated with 3TM at different concentrations, ranging from 1 to 100 µM, for 3 days using the SRB assay (Figure 2A and B). In the presence of different doses of 3TM, the cells were inhibited ranging from 10 to 90% with a loss of viable cells (Figure 2C and D). 3TM inhibited the proliferation of MCF-7 and HCT-15 cells in a concentration and time-dependent manner. The IC_{50} was determined by

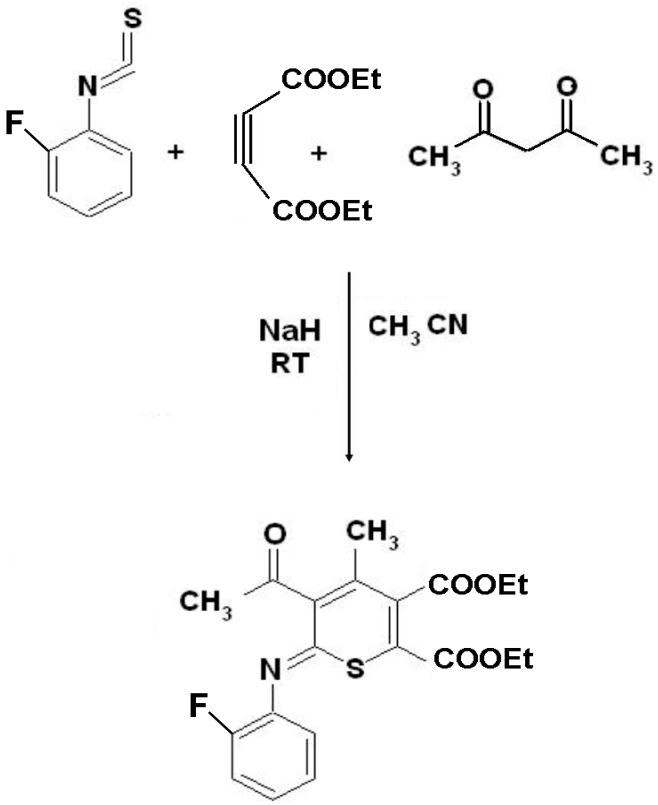

Figure 1. Synthesis of diethyl 5-acetyl-4-methyl-6-(2-fluorophenylimino)-6H-thiopyran-2,3-dicarboxylate.

interpolation from dose-response curves. The percentage growth inhibition was calculated by comparison of the absorbance of treated versus control cells. IC_{50} calculated for HCT-15 was 4.5 and 7 µM for MCF-7 (Figure 2C and D). Control group showed regular polygonal shape, and cell antennas were short. The cell morphology of treated cells was affected by 3TM treatment; loss of adhesion, rounding, cell shrinkage and detachment from the substratum were observed.

Clonogenic assays

Analysis of clonogenic activity in HCT-15 and MCF-7 cells treated with 3TM at concentrations ranging from 0.5 to 100 µM of 3TM for 14 days revealed a complete inhibition of colony formation at 3 µM for HCT-15 and 5 µM for MCF-7 cells, whereas concentrations lower than 10 µM were ineffective (Figure 2E and F).

The percentage of growth inhibition was calculated by using the equation: $(1- A_t/A_c) \times 100$, where A_t and A_c represent the absorbance in treated and control cultures, respectively. IC_{50} was determined by interpolation from dose-response curves. Dose-response curve of 3TM mediated inhibition of HCT-15 (E) and MCF-7 (F) cell colony formation.

DNA binding study

DNA as a carrier of genetic information is a major target for drug interaction because of the ability to interfere with transcription (gene expression and protein synthesis) and DNA replication, a major step in cell growth and division. The calculation of the overall binding constants was carried out on the basis of UV absorption as reported. The equilibrium for 3TM and DNA complex can be described as follows:

$$[DNA + 3TM] \leftrightarrow [DNA - 3TM]K_{3TM}$$

$$K_{3TM} = [DNA - 3TM]/ [DNA][3TM]$$

The double reciprocal plot of $1/[A - A_0]$ vs 1/[ligand] is linear and the association binding constant (K) is calculated from the ratio of the intercept on the vertical coordinate axis to the slope (Tajmir-Riahi et al., 1995; Mandeville et al., 2010; Froehlich et al., 2011). Concentrations of the complexed ligand were determined by subtracting absorbance of free DNA at 260 nm from those of the complexed DNA. Concentration of the free ligand was determined by subtraction of complexed ligand from total ligand used for the experiment. Our data shows that 1/[complexed ligand] almost proportionally

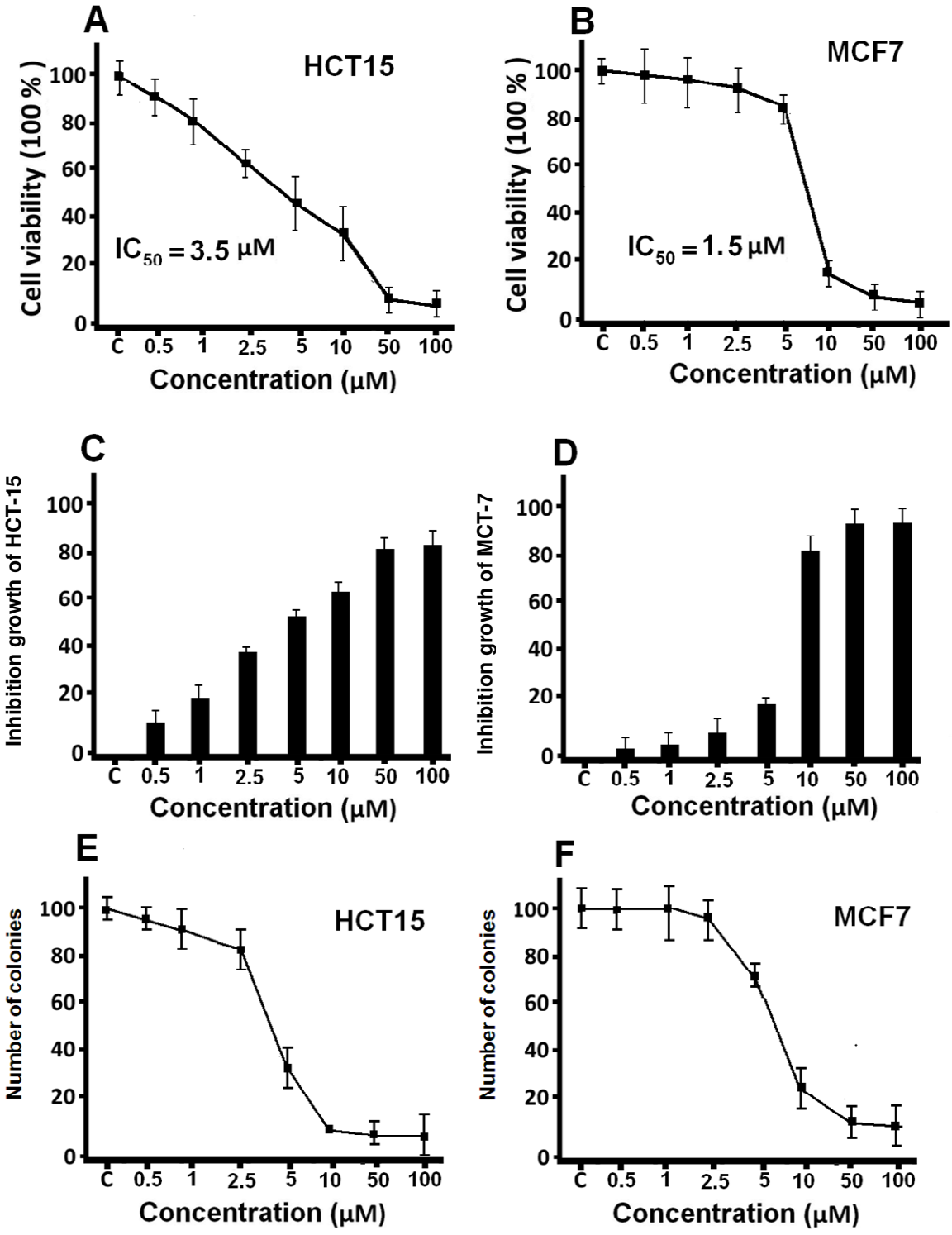

Figure 2. Effects of 3TM on the proliferation of HCT-15 and MCF-7. (A) Effect of 3TM on the proliferation of HCT-15; (B) effects of 3TM on the proliferation of MCF-7; (C) inhibition of 3TM on HCT-15 growth; (D) inhibition of 3TM on MCF-7 growth.

increases as a function of 1/[free ligand] (Figure 3). Therefore, an overall binding constant, $K_{3M\text{-}DNA}$ = 2.4 x 10^4 M^{-1} can be estimated for 3TM –DNA.

Conclusion

We reported here that the compound diethyl-5-acetyl-4-

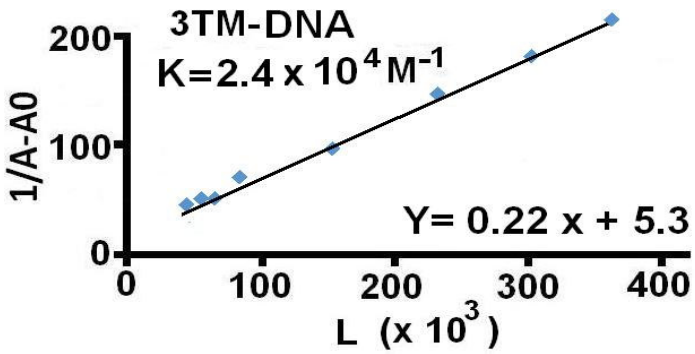

Figure 3. The plot of $1/(A-A_0)$ vs. 1/L for DNA and iPA complexes at different drug concentrations.

methyl-6-(2-fluorophenylimino)-6H-thiopyran-2,3-dicarboxylate was synthesized and characterized. The antiproliferative effects mediated by 3TM on the growth of HCT-15 and MCF-7 cell lines, monitored at different concentrations, ranging from 1 to 100 µM for 3 days and IC_{50}, calculated by SRB assay, were 4.5 µM for HCT-15 and 7 µM for MCF-7. This study of these two human cancer cell lines revealed suppression of clonogenic activity after exposure to 3TM at a concentration of 4 µM for HCT-15 and of 5 µM for MCF-7. On the other hand, the connection between DNA binding and cytotoxicity of 3TM is supported by a large body of literature. Our result from structural analysis showed interaction of 3TM with DNA and the binding constant value $K_{3M\text{-}DNA} = 2.4 \times 10^4$ M^{-1} suggest that 3TM interacts with DNA in good mode. Further research is required in order to demonstrate whether this binding effect may be related to DNA damage or apoptosis in both cancer cells.

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