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Research Article

INHIBITION OF HUMAN MCF-7 BREAST CANCER GROWTH BY FREE RADICALS ENHANCEMENT OF POLYCONDENSED THIENOPYRIMIDINE DERIVATIVES

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ABSTRACT

In our previous work some novel indeno[2,1-*b*]thiophenes, indeno[1′,2′:4,5]thieno[2,3-*d*][1,2,3]triazines, indeno[1′,2′:4,5]thieno[2,3-*d*]pyrimidines, indeno[1′,2′:4,5]thieno[2,3-*d*][1,3]thiazolo[3,2-*a*]pyrimidines, and indeno[1′,2′:4,5]thieno[2,3-*d*][1,2,4]triazolo[4,3-*a*]pyrimidines **2–16** were prepared starting with 2-aminoindeno[2,1-*b*]thiophene-3-carboxylic acid amide **(1)**. Furthermore, certain of these compounds exhibited antimicrobial activity. The objective of this study was to evaluate the anticancer activity of the prepared compounds against human breast cancer cell line MCF-7, and the results were compared with the activity of these compounds with the control untreated cells. Treatments of MCF-7 cells with 1/10 of IC_{50} of the prepared compounds as determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay (using gradually doses 5, 10, 25, 50 or 100 µg/ml) revealed that many of them revealed promising anticancer activity. Furthermore, the activity of superoxide dismutase (SOD) and the levels of hydrogen peroxide (H_2O_2), nitric oxide (NO) and lipid peroxidation (LP) were significantly increased, while the activities of catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GSH-R) and the level of reduced glutathione (GSH) were significantly lowered with decline the total protein, DNA and RNA content compared with MCF-7 cells harvested from control. Moreover, the result indicated significant inhibition of cell growth by these compounds through inhibition of cytochrome P-450 enzymes, which are needed in the activation of carcinogen. This was partially demonstrated by the aniline hydroxylase inhibition data. The results suggested that the prepared compounds possess significant anticancer activity through regulation of free radicals generation and may be potent anticancer agents for inclusion in modern clinical trials.

Keywords: Thienopyrimidine; Antioxidant; Anticancer; MCF-7 cell line.

INTRODUCTION

Numerous compounds with biological activity has been investigated, however many of them are not suitable for therapeutic use due to their toxic, carcinogenic and mutagenic properties. The use of chemotherapeutic drugs in cancer therapy involves the risk of life threatening host toxicity. The search therefore continues to develop the drugs which selectively act on tumor cells[1]. So, discovery of the anticancer activity of the chemical synthetic compounds and its successful clinical use represents important progress for inorganic medicinal chemistry[2].

The fused thieno[2,3-*d*]pyrimidine derivatives represent one of the most active class of compounds owing to their synthetic and effective biological importance[3]. They bear structure analogy and isoelectronic relation to purine and several substituted thieno[2,3-*d*]pyrimidine derivatives were shown to exhibit prominent and versatile biological activities[4]. Recently, many of their derivatives have been synthesized as potential analgesic[5], antimicrobial[6] and antiviral agents[7].

As a result of many extensive studies in the field of anticancer drugs, different types of anticancer chemicals with promising effects have emerged such as anthracylines, cisplatin and alkylating agents that induce apoptotic cell death in cancer cells via generation of reactive oxygen species (ROS)[8]. ROS cause cellular disruption due to peroxidation of membrane lipids. ROS include three major radical species: superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\cdot}OH$)[9].

In the previous work some novel indeno[2,1-b]thiophenes, indeno[1′,2′:4,5]thieno[2,3-*d*][1,2,3]triazines, indeno[1′,2′:4,5]thieno[2,3-*d*]pyrimidines, indeno[1′,2′:4,5]thieno[2,3-*d*][1,3]thiazolo[3,2-*a*]pyrimidines, and indeno[1′,2′:4,5]thieno[2,3-*d*][1,2,4]triazolo[4,3-*a*]pyrimidines **2–16** were prepared starting with 2-aminoindeno[2,1-*b*]thiophene-3-carboxylic acid amide **(1)** Furthermore, the antimicrobial evaluation of the prepared products showed that many of them revealed promising antimicrobial activity[10]. In this context, we will try to elucidate the mechanism whereby the prepared compounds exert their anticancer activity and propose that the anticancer effects of these compounds stem from the fact that they able to regulate the free radical balance. So, we estimated the growth-inhibitory activities of these compounds on human MCF-7 breast cancer cells line and also evaluated the activities of some free radicals enzymes including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GSH-R) and oxidative stress parameters hydrogen peroxide (H_2O_2), nitric oxide (NO), reduced glutathione (GSH), lipid peroxidation (LP) in MCF-7 cells treated with prepared compounds with estimation the effect of these compounds on the level of total protein and nucleic acids.

MATERIALS AND METHODS

Chemicals

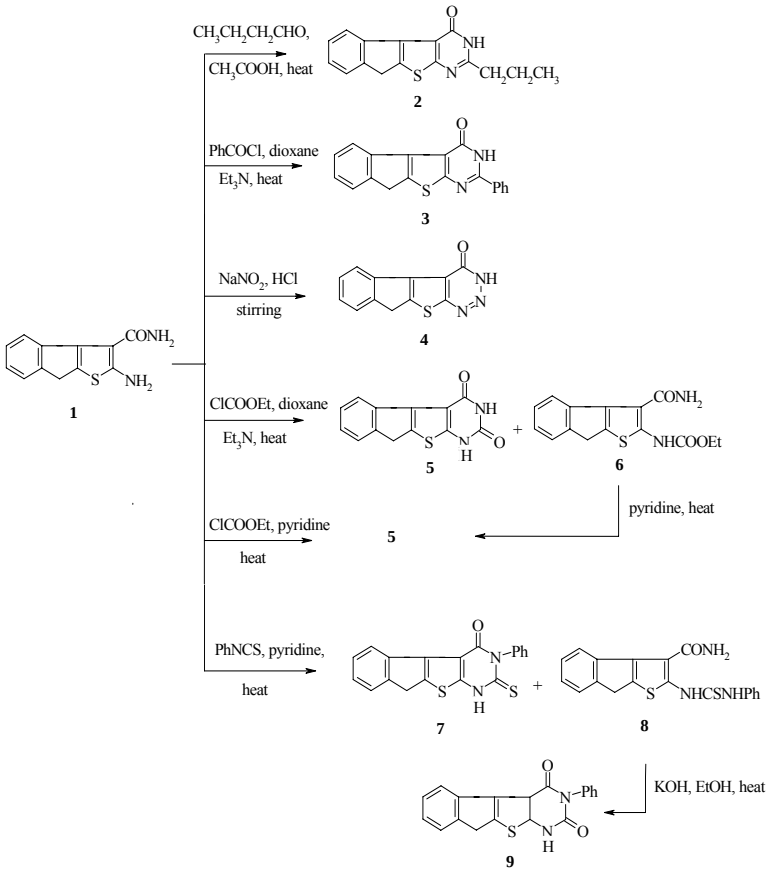
Dimethylsulphoxide (DMSO), phenobarbital and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were purchased from Merck (Darmstadt, Germany). All other chemicals and reagents used in this study were of analytical grade and purchased from Sigma-Aldrich chemical Co. (St. Louis, MO, USA).

Animals

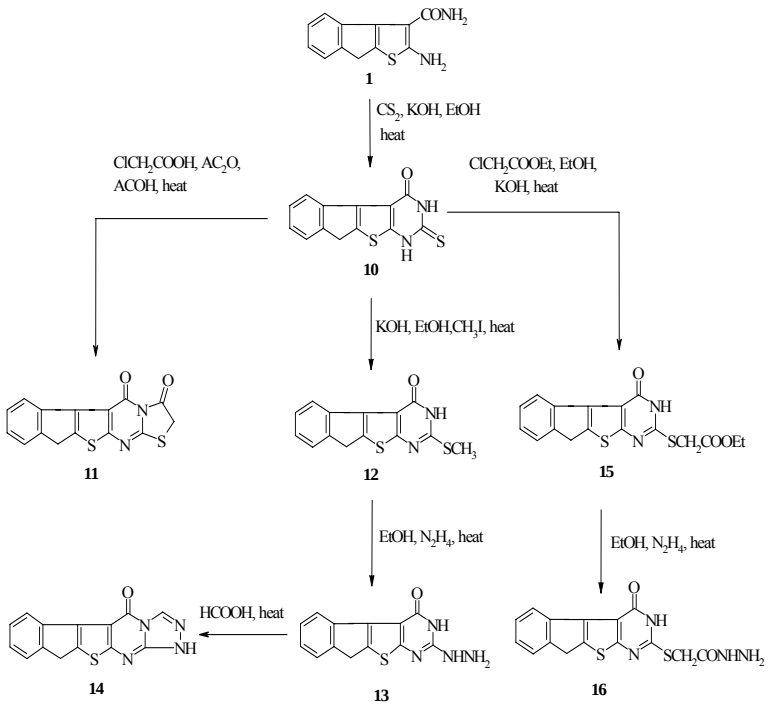
The animal care and handling was done according to the guidelines set by the World Health Organization, Geneva, Switzerland. Healthy, and according to the committee for animals care at the National Research Centre, Egypt. Adult male Sprague-Dawley rats with an average body weight of 170 ± 20 g were obtained from the animal house of National Research Centre, Egypt. The animals were housed under standard laboratory conditions (12 h light / 12 h dark) in a room with controlled temperature (24±3 °C). The rats were fed with standard commercial rat chow and drinking water.

Cell Culture

About 0.50 x 10^6 cells of MCF-7 human breast cancer cell line were maintained and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (GIBCO), penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37 ^{o}C in humidified atmosphere containing 5% CO_2.



Scheme 1



Scheme 2

***In Vitro* Cytotoxicity Assay**

For *in vitro* short term cytotoxicity evaluation of the prepared compounds (Scheme 1 and 2) as previously prepared[10], MCF-7 cells were plated in a concentration of 0.65 x 10^5 cells per well, in complete culture medium in 96 – well flat – bottomed culture plates (Falcon) for 24 h to assure total attachment. Then 20 µl of various concentration of test compounds (5, 10, 25, 50, or 100 µg/ml) were added to the cells suspended in 0.10 ml of phosphate buffered saline (PBS) (0.20 M, pH 7.4), the control cells without the test compounds were also cultured, then the plate was incubated for 24 h at 37 °C, in a humidified 5% CO_2 atmosphere. Cells survival was evaluated at the end of the incubation period with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay according to Vistica et al.[11].

This test is based on the selective ability of living cells only to reduce the yellow soluble salt of MTT to a purple-blue insoluble formazan precipitate. The viable cell number is proportional to the production of formazan salts. The crystals of formazan were dissolved in 10 % DMSO and the optical density was measured spectrophotometrically. After incubation, media were removed and 40 µl MTT solution/well were added and incubated for an additional 4 h. MTT crystals were solubilized by adding 200 µl of 10 % DMSO/well and plate was shaken gently for 10 min at room temperature. The absorbance was determined photometricaly at 570 nm using microplate ELISA reader (Microplates reader, Asys Hitech, Austria), where the optical density is directly proportion to the number of living cells in the culture. The experiments were performed in six replicates for each compound and the results were normalized to the control value i.e. [100 x (Absorbance of sample - Absorbance of control / Absorbance of control)] and expressed as percentage of control.

The compound concentrations which give 50% growth inhibition are referred to as the IC_{50} and values were obtained mathematically from the concentration response curve using a computer program for probit analysis. After that, the cells in culture medium were treated with 20 µl of 1/10 of IC_{50} values of the compounds, then incubated for 24 h at 37 °C, in a humidified 5% CO_2 atmosphere. The MCF-7 cells were harvested and homogenates were prepared in saline using a tight pestle homogenizer until complete cell disruption for further biochemical analysis.

Antioxidant enzyme assays

The supernatant obtained after centrifugation of cell homogenates was used for determination of enzymes activities including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and glutathione reductase (GSH-R) as described by Paglia and Valentine[12], Aebi[13], Marklund and Marklund[14], Carlberg and Mannervik[15] respectively.

Oxidative stress assays

The levels of hydrogen peroxide (H_2O_2), nitric oxide (NO), lipid peroxidation (LP) indicated as thiobarbituric acid reactive substances (TBARS) and reduced glutathione (GSH) were determined by the method of Wolf[16], Granger et al.[17]; Conrad et al.[18] and Ellman[19] respectively.

Estimation of nucleic acids and protein levels

Nucleic acids (DNA and RNA) and total protein were precipitated and measured in cell homogenates. Total DNA was extracted and assayed according to the method described by Zhou et al.[20], total RNA was extracted and assayed according to the method adopted from the method provided by Hybaid/AGS (Germany), and total cellular protein was assayed by the method of Lowry et al.[21].

Aniline hydroxylase inhibitory assay

Aniline hydroxylase assay was performed by the method described by Mazel[22]. Where the enzyme was induced in rats by the oral administration of phenobarbital (80 mg / Kg) for 5 days. Then 10% of liver homogenate was used for assay. Various concentrations of each compound were added to the reaction mixture and incubate at 37 °C. *p*-aminophenol formed during the enzyme action reacts with phenol to form a blue colored product, which was measured at 630 nm. The percentage inhibition of the enzyme was calculated by comparing the absorbance of control and that of compounds.

Statistical analysis

The results were reported as Mean ± Standard error (S.E.) for at least six times experiments and analyzed statistically using a one-way analysis of variance (ANOVA) followed by the student *t*-test for comparison between groups, wherein the differences were considered to be significant at $p < 0.05$.

RESULTS AND DISCUSSION

The viability assay which measure the metabolic capacity of the prepared compounds was applied with a broad range of concentrations as is usual when starting to examine the cytotoxicity of unknown compounds or drugs[23]. The cytotoxicity of the tested compounds was evaluated in MCF-7 cell line is expressed in terms of relative cell viability of compounds – treated cells in comparison to control cells for a duration of 24 h exposure to tested compounds. The percentage of cytotoxicity was calculated considering the control as 100% cell viability.The results indicated that cytotoxicity represented by median growth inhibitory concentration (IC_{50}) which required to produce 50% cytotoxic effect of MCF-7 cells after 24 h exposure to tested compounds was 45, 6, 7, 66, 14, 15, 36, 29, 19, 23, 21, and 46 µg / ml for compounds1, 4, 5, 6, 7, 8, 9, 11, 12, 13, 14 and 15 respectively (Figure. 1). It is observed that there was gradual decrease in the viability of cancer cells with increasing the concentration of the tested compounds in a dose-dependent inhibitory effect. Therefore, these compounds have antitumor activities. DMSO did not seem to have any noticeable effect on cellular growth. Though the mode of action of tested compounds are still unclear. The results showed that the order of anticancer activity was: 4 > 5 > 7 > 8 > 12 > 14 > 13 > 11 > 9 > 1 > 15 > 6.

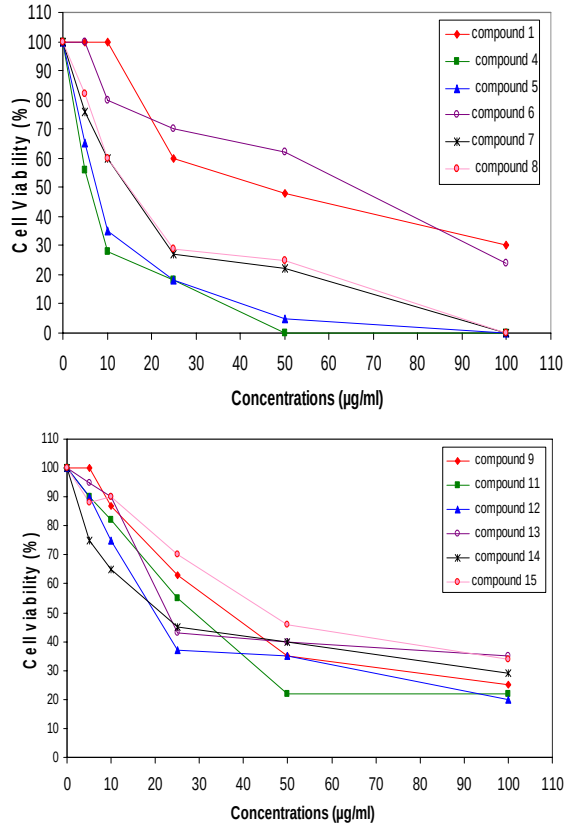


Fig. 1: Effect of treatment at various concentrations of prepared compounds on MCF-7 cell line cytotoxicity (IC_{50}) as measured with MTT method

To elucidate the mechanism by which the prepared compounds exert their anticancer activities, we estimated the activities of free-radicals-metabolizing enzymes (SOD, CAT, GSH-Px, GSH-R); the levels of (GSH, H_2O_2, NO, LP, total protein, RNA, DNA) as well as the activity of aniline hydroxylase. As shown in Table 1 in general treatment of the cells with 1/10 of IC_{50} values of the prepared compounds resulted in increase in the activity of SOD higher than those of control accompanied with depletion in the activity of CAT, GSH-Px, and GSH-R, as well as the level of GSH. On the other hand, the data revealed that the levels of H_2O_2, NO and LP were higher than those of control.

These results indicate that the antitumor effect of the present compounds may be exerted at least partly by production of H_2O_2.

Table 1: Percentage change in the activity of SOD, CAT, GSH-Px, GSH-R enzymes and GSH, H_2O_2, NO, LP levels caused by prepared compounds as compared with control in MCF-7 cell line

Compound NO.	SOD	CAT	GSH-Px	GSH-R	GSH	H_2O_2	NO	LP
1	+33	NS	-11	-7	NS	+30	+10	+36
4	+160	-66	-67	-92	+95	+20	+165	+120
5	+120	-60	-62	-82	+85	+18	+142	+90
6	+45	-21	-12	NS	+35	+25	+19	NS
7	+46	-19	-9	-22	+15	+33	+25	+43
8	+110	-50	-54	-14	+80	+170	+ 55	+73
9	+55	-12	-14	-32	NS	+38	NS	+27
11	+85	-31	-30	-55	+45	+111	+60	+65
12	+92	-36	-36	-33	+62	+125	+43	+60
13	+36	-22	-6	-20	NS	+24	NS	+25
14	+12	-13	NS	-25	+19	+26	+15	NS
15	+25	-19	NS	NS	+21	+19	NS	+43

(+) increased vs. control, (-) decreased vs. control, (NS) No significant change vs. control

The antitumor activities of these compounds may be accompanied by increases in SOD activities of tumor-treated cells compared to control group. This means that these compounds can cause H_2O_2 production. The H_2O_2 produced should be rapidly removed through the activation of CAT, GSH-Px and GSH-R. The present results show that activities of CAT, GSH-Px and GSH-R and the level of GSH are lowered in groups treated with compounds compared to control group (Table 1). Consequently, the excess H_2O_2 produced in tumor cells with these compounds can not be removed. In other words, the accumulation of H_2O_2 in tumor cells should be partly the cause of tumor cell killing. Thus the results of the present study are consistent with the hypothesis that the prepared compounds exert their antitumor effects because they produce ROS.

As shown in Table 2, nucleic acid and protein concentrations were significantly lowered in MCF-7 cells treated with all compounds at the 1/10 of IC_{50} values of the compounds as compared to control. The prepared compounds also showed a dose dependent inhibition of aniline hydroxylase. Concentration required 50% inhibition of aniline hydroxylase was found to be 46, 32, 21, 42, 36, 60, 46, 19, 22, 61, 40, 65 µg / ml for compounds1, 4, 5, 6, 7, 8, 9, 11, 12, 13, 14, and 15 respectively (Figure. 2).

Table 2: Percentage change in the levels of total protein, RNA and DNA caused by prepared compounds as compared with control in MCF-7 cell line

Compound No.	Protein	RNA	DNA
1	- 13	- 19	- 25
4	- 95	- 90	- 68
5	- 90	- 77	- 60
6	- 35	NS	- 23
7	- 26	- 33	- 33
8	-32	- 83	- 64
9	- 35	- 28	- 16
11	- 95	- 76	- 71
12	- 22	- 65	- 55
13	- 16	- 32	NS
14	- 22	NS	NS
15	-35	- 29	- 18

(+) increased vs. control, (-) decreased vs. control, (NS) No significant change vs. control

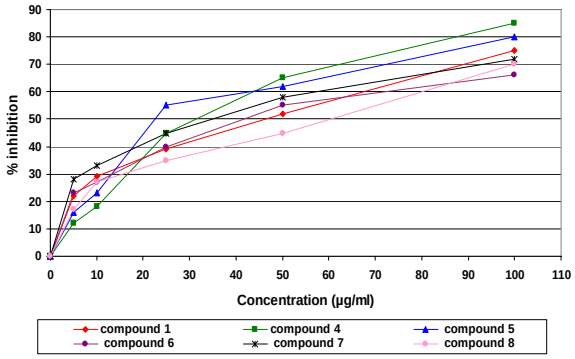


Fig. 2: Aniline hydroxylase inhibitory activity of prepared compounds

Moreover, the results showed that treatment with these compounds lead to an increased in the level of NO (especially compounds **4** and **5**) with decrease in the level of total protein, RNA, DNA which can be explained according to Blanco et al.[24] who mentioned that an increase in NO level leads to apoptosis, whereas an increase in ROS leads to necrosis, so, the way a tumor cell dies reflects the radical balance in the system. NO has several cytotoxic effects, including reactions with proteins and nucleic acids. The main targets of NO in proteins are the SH group[25] and Fe of active sites[26]. In the nucleus, NO has been shown to cause mutations of genes[27], and inhibition of DNA repair enzymes[28], and mediate DNA strand breaks[29]. NO has been shown to mediate apoptosis[30].

Our results are consistent with Bienvenu et al.[31] who reported that most chemotherapeutic agents cause cells to over generate (ROS) and, thus, are capable of inducing apoptosis and necrosis causing oxidative damage to DNA, proteins and lipids. Moreover, the cascade of signals mediating apoptosis often involves a ROS intermediate messenger, and ROS can short circuit the pathway, bypassing the need for upstream signals for cell suicide. Recently, Huang et al.[32] reported that regulation of free radical-producing agents may also have important clinical applications. This mechanism for the effects of ROS generating anticancer agents is only beginning to be understood, as previously the mechanism of most anticancer agents was believed to be due mainly to direct interaction with DNA and interference with DNA regulatory machinery (e.g., topoisomerases and helicases) and to the initiation of DNA damage via production of ROS[33]. Moreover, these data indicate significant inhibition of cell growth by these compounds. How this activity is related with carcinogenic activity of the examined compounds is not known at present. The compounds were found to inhibit cytochrome P-450 enzymes, which are needed in the activation of carcinogen. This was partially demonstrated by the aniline hydroxylase inhibition data (Figure, 2).

CONCLUSIONS

In conclusion, the present results suggest that the prepared compounds possess antitumor activity and they exert their antitumor activities partly by increasing free radicals production and by depletion of intracellular catalase, glutathione peroxidase, glutathione reductase, reduced glutathione. The results revealed that these compounds may be potent anticancer agents for inclusion in modern clinical trials.

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