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# Oxidative Stress of Some Triazolopyrimidine Derivatives and their Nucleoside Analogues on Mcf-7 and A549 Cell Lines

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THE synthesis and assessment of the biological benefits and applications of new triazolopyrimidines and their glycoside derivatives were discussed and researched thoroughly. Interestingly, the data revealed activity of these newly synthesized compounds in A549 and MCF-7 cell lines, where MCF-7 cells responded more effectively to the examined compounds than the other cell lines. The present work's aim is to appraise the behavior of some free radical enzymes, including SOD, CAT, GSH-Px and oxidative stress parameters $H_2O_2$, NO, GSH in MCF-7 and A549 treated cells. The results brought out that, there is an increase in the level of $H_2O_2$ and NO associated with an acme in the SOD enzyme activity, besides, there is a diminution in the activities of CAT, GSH and GSH-Px when we compared it with control. These results may indicate that compounds 1, 2, 3 and 4 may exert their anticancer activities - to some extent – through the inflection of both the production of ROS and the action of antioxidant enzymes.

**Keywords:** Triazolopyrimidines, Cancer, Oxidative stress, Anti-oxidants, MCF-7, A549

## Introduction

Cancer diseases represent one of the most common causes of mortality among the different causes of death all over the world. Many patients are suffering from the complications related to the disease development and different treatment side effects. The challenge of discovering new chemotherapeutic substances to provide a better treatment for different types of cancer represents a great rivalry among scientific researchers. Many attempts were made in many fields trying to discover new compounds which may have anti-proliferative effect on cancer cells and may be able to be used as chemotherapy in cancer treatment. Searching in heterocyclic compounds and its synthesis are important tools for developing a new treatment and get many potent biologically effective compounds [1-4].

The pyrimidine ring represents one of the most abundant systems in life as monocyclic or ring merged compounds and thus found in many important biological systems, such as the nitrogenous bases in the DNA and RNA; cytosine, thymine and uracil, vitamin $B_1$ and alloxan. Also, it can be an important heterocyclic core in multiple synthetic molecules like barbiturates and drugs, such as zidovudine; the HIV drug [5-7].

Purines are an additional group of heterocyclic compounds, it can be found in high concentrations in many dietary sources such as red animal protein and manufactured meat products, particularly the internal organs like liver and kidney. As a common rule, vegetables and vegetable rich meals have a small concentration level of purines [8]. Examples of purine rich sources include: scallops, cow kidneys, brains, liver, herring, mackerel, anchovies and sardines [8, 9]. The two essential nitrogenous

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bases which represent an important constituent in the synthesis of DNA and RNA are purines and pyrimidines. With the intention of forming of DNA and RNA, both pyrimidines and purines are required by the cell in more or less the same quantities. Both purine and pyrimidine are under negative or positive feedback control mechanisms which regulate their production, when purines are formed, they reduce the enzymatic activities of the enzymes necessary for more purine synthesis. This negative feedback occurs since they are also needed for the activation of the enzymes responsible for pyrimidine production. Pyrimidine at the same time self-inhibits and activates purine in a similar pathway. As a result of this, there is almost the same quantity of both compounds within the cell at all times [6]. Since pyrimidines and purines play a pivot role in the biological system - particularly in the nucleic acids - many researchers focus on its derivatives as promising molecules with biological activities which can be used to reduce suffering of many diseases, many authors indicated that incorporation of another aryl or hetero-aryl ring to the pyrimidines core can make changes in its biological activities [10-13].

In a latter study, the *N*-glycosides and thioglycoside derivatives of the triazolopyrimidine ring system as well as their analogs possessing acyclic sugars were synthesized [14]. Recently, Mohamed et al. reported the synthesis of some new novel substituted triazolopyrimidine derivatives by using triazolo-carboxamide compound as the key precursor [15]. Their cytotoxic effect and anti-tumor assessment were tested in three different types of human cancer cell lines which are lung A549, breast MCF-7 and colon HCT116 in vitro. The obtained data, specify that these compounds bring their effect in MCF-7 and A549, where the first are more receptive to the prepared candidates than the other cell lines. In this context, we will attempt to clear up the pathway (pathways) through which the prepared compounds can bring on their anti-proliferative achievement. We suggest that the anticancer impact of the examined compounds arise from the information that they may be capable to exchange the free radical equilibrium. Therefore, we evaluate the activities of some free radical enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and some oxidative stress parameters like hydrogen peroxide ($H_2O_2$), nitric oxide (NO), reduced glutathione (GSH) in MCF-7 and A549 treated cells.

## Expreimental

*Chemicals:*

Fetal bovine serum (FBS) and L-glutamine, both of them were purchased from GIBCO Invitrogen Company (Scotland, UK). Dulbecco's modified Eagle's (DMEM) medium was obtained from Cambrex (New Jersey, USA)., Doxorubicin, penicillin, streptomycin and dimethyl sulfoxide (DMSO) all these products were obtained from Sigma Chemical Company (Saint Louis, MO, USA).

*Cell lines and culturing:*

Human breast MCF-7 and lung A549 cancer cell lines were the two types of human tumor cell lines involved in this study; they were obtained from the American Type Culture Collection (Rockville, MD, USA). The tumor cells were preserved at 37ºC in Dulbecco's modified Eagle's medium (DMEM) provided with penicillin (100 U/ml), streptomycin (100 µg/ml) and 10% heat inactivated fetal calf serum (GIBCO), in a wet condition containing 5% $CO_2$. In a 25 $cm^3$ flask a concentration of 0.50 x $10^6$ of cells were incubated and allowed to stand in 5 ml of complete culture medium [16]. The cells in culture medium were handled with a volume of 20 µl of their 1/10 $IC_{50}$ values of the examined compounds 1, 2, 3 and 4 respectively, and the standard reference anti cancer drug; doxorubicin as described in our previous work, then incubated for 24 h at 37ºC, in a moistured atmosphere with 5% $CO_2$. The A549 and MCF-7 cells were collected and the homogenates were made in a saline using a tightly pressing homogenizer till the complete cell rupture. The supernatant of cell homogenates obtained after centrifugation was used for determination of the following parameters; enzyme activities superoxide dismutase (SOD); the principle of this assay depends on its ability to inhibit the reaction of phenazine methosulphate-mediated with nitroblue tetrazolium dye [17, 18]. Catalase (CAT); the enzyme allows to react with a known volume of $H_2O_2$, and then the reaction is inhibited after exactly one minute with catalase inhibitor .

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

$$2H_2O_2 + \text{DHBS} + \text{AAP} \xrightarrow{\text{HRP}} \text{Quinoneimine Dye} + 4H_2O$$

Then the remaining $H_2O_2$ reacts with 3,5-Dichloro -2-hydroxybenzene sulfonic acid (DHBS) and 4-aminophenazone (AAP) in the presence of peroxidase (HRP) as a catalyst, the intensity of the produced color is inversely proportional to the activity of the enzyme in the

original sample [19, 20].

glutathione peroxidase (GSH-Px); The main principle of the assay depends on the indirect measure of the activity of GSH-Px, the oxidized glutathione (GSSG) - produced during the reduction of a peroxide compound by GSH-Px – is allowed to convert to its reduced state through the action of the enzyme glutathione reductase (GR):

$$\text{R-O-O-H} + 2\text{GSH} \xrightarrow{\text{GSH-Px}} \text{R-O-H} + \text{GSSG} + \text{H}_2\text{O}$$

$$\text{NADPH} + \text{H+ GSSG} \xrightarrow{\text{GR}} 2\text{GSH} + \text{NADP}^+$$

The decrease in absorbance at 340nm (A340) during the oxidation of NADPH to $NADP^+$ gives us an indication about the enzyme activity. To assay GSH-Px, a cell or tissue homogenate is gathered with a solution including glutathione, glutathione reductase, and NADPH. The enzyme reaction is started by adding the substrate, hydrogen peroxide and at a wavelength of 340 nm the absorbance (A340) is recorded. The rate of decrease in the A340 is directly proportional to the GSH-Px activity in the sample [21, 22].

The level of hydrogen peroxide ($H_2O_2$); Measuring of hydrogen peroxide ($H_2O_2$) depend on that $H_2O_2$ reacts with 3,5-dichloro-2-hydroxybenzensulfonic (DHBS) acid and 4-aminophenazone (AAP) in the presence of peroxidase (HRP), to produce a chromophore.

$$2\text{H}_2\text{O}_2 + \text{DHBS} + \text{AAP} \xrightarrow{\text{HRP}} \text{Quinoneimine Dye} + 4\text{H}_2\text{O}$$

The level of nitric oxide (NO); Since nitrite ($NO_2^-$) and nitrate ( $NO_3^-$ ) represents the final products of NO in vivo, the principal of this method depend on the addition of Griess Reagents which change nitrite into a deep purple azo compound, measuring of the absorbance of this formed color accurately determines $NO_2^-$ concentration. Nitrites react with Sulphanilamide in an acid medium to produce nitrous acid diazotise sulphanilamide which, coupled with N-(1–naphthyl) ethylenediamine, the reddish–purple color produced from this azo dye reaction can be measured at 540 nm [23, 24].

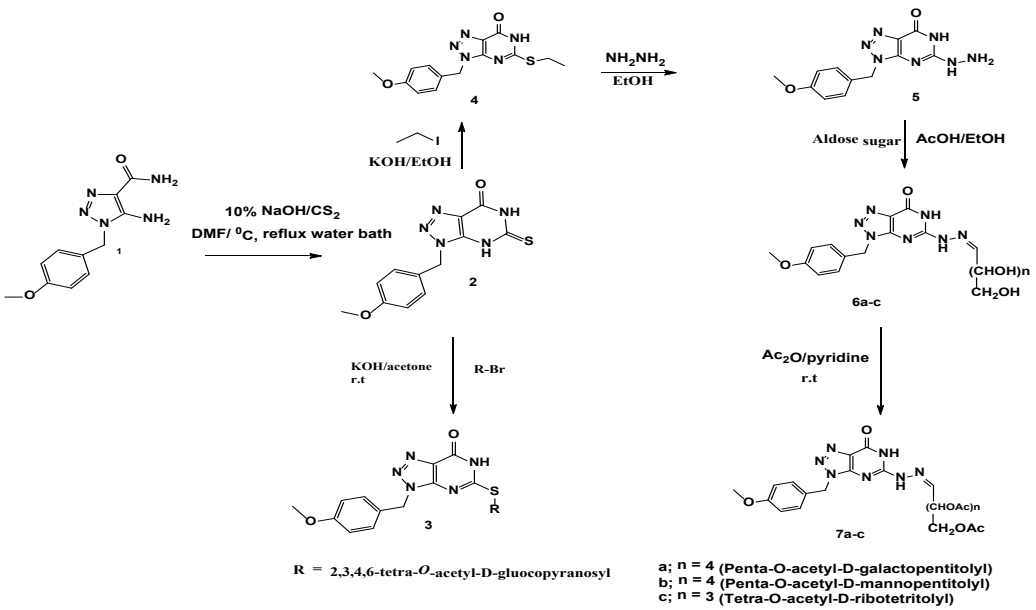
Reduced glutathione (GSH); the main idea of this method depends on the reduction of 5,5` dithiobis (2- nitrobenzoic acid) (DTNB) with glutathione (GSH) to make a yellow compound. Measuring of absorbance at wavelength 405 nm give the concentration of GSH, which is directly proportional to the intensity of the formed color [25-27]. Total cellular protein was screened according to the procedure of [28, 29].

*Statistical analysis of the data*

The outcomes are conveyed as Mean ± Standard error (S.E.) for at least four times experiments. Statistical differences were examined by one way ANOVA test followed by the student's t-test where results considered to be significant at $p < 0.05$.

## Results and Discussion

In our recent reported results novel functioned triazolopyrimidine heterocycles were prepared via heterocyclization of the 5-amino-1-(4-methoxybenzyl)-1*H*-1,2,3-triazole-4-carboxamide (**1**). Reaction of the carboxamide (1) with carbon disulfide in the presence of 10% sodium hydroxide afforded 3-(4-Methoxybenzyl)-5-thioxo-5,6-dihydro-3*H*-[1,2,3]triazolo[4,5-d] pyrimidin-7(4*H*)-one, product **(2)** in 77% yield. The obtained triazolopyrimidine thione derivative (**2**) were converted to their derived thioglycoside 5-(2,3,4,6-Tetra-O-acetyl-D-glucopyranosylthio)- 3-(4-methoxybenzyl)-3*H*- 1,2,3]triazolo[4,5-d] pyrimidin-7(6*H*)-one (**3**) by the reaction with glucosyl bromide in the presence of potassium hydroxide . Furthermore, Alkylation of the triazolopyrimidine thione (2) with ethyl iodide in alkaline medium afforded **5**-(Ethylthio)-3-(4-methoxybenzyl)-3*H*-[1,2,3] triazolo[4,5-d]pyrimidin-7(6*H*)-one (**4**) in 74% yield. Hydrazinolysis of the produced S-ethyl compound gave the required 5-Hydrazinyl-3-(4-methoxybenzyl)-3*H*-[1,2,3]triazolo[4,5-d] pyrimidin-7(6*H*)-one (**5**) in good yield. Linked acyclic sugar moieties as acyclic nucleoside analogs were also synthesized when the hydrazine derivative 5 was allowed to react with a number of monosaccharides, namely D-galactose, D-mannose and Dribose in an aqueous ethanolic solution and with catalytic amount of acetic acid, the corresponding hydrazinyl sugar derivatives namely, (Z)-3-(4-Methoxybenzyl)-5-(hydrazinylsugar)-3*H*-[1,2,3]triazolo[4,5-d]pyrimidin-7(6*H*)-one (**6a-c**) were obtained in 75-79% yields. Acetylation of compounds 6a-c with acetic anhydride in pyridine at room temperature lead to the formation of per-*O*-acetylated derivatives namely (Z)-3-(4-Methoxybenzyl)-5-(per-O-acetylhydrazinylsugar)-3*H*-[1,2,3]triazolo[4,5-d]pyrimidin-7(6*H*)-one (**7a-c**), respectively, in 80% yield (Scheme 1)[15]. The prepared glycosides and acyclic investigated for their anticancer activities against human breast MCF-7 and lung A-549.




**Scheme 1: Synthesis of triazolopyrimidine sugar derivatives**

We found that the examined compounds have an anti-carcinogenic action in both lung A549 and breast MCF-7 cancer cell lines via the inhibition cell reproduction and showed considerable reducing of the cell proliferation, especially, compounds **1, 2, 3 and 4** which shows potential anticancer effect when it's put side by side with the generally used drug used in the treatment of different types of cancers, doxorubicin. The experimental data indicated that MCF-7 cells are extra susceptible to the evaluated molecules than A549 cells [15].

To explicate the pathways by which these compounds may produce their anticancer effects, we predicted the activities of the enzymes responsible for the metabolizing of the free radicals; SOD, CAT and GSH-$P_X$, plus an evaluation of the levels of $H_2O_2$, NO and GSH as oxidative stress parameters in both MCF-7 and A549 cells exposed to the tested compounds.

As illustrated in (tables 1-4), in the general treatment of A549 and MCF-7 cells with compounds 1, 2, 3 and 4 with the tenth of their half maximal inhibitory concentration ($IC_{50}$) values or doxorubicin revealed a considerable rise in SOD activity and the concentration of $H_2O_2$ and NO more than their elevation in the control group, escorted with a significant drop in the activity of both CAT and GSH-Px, and GSH level. This indicates that these compounds' anticancer activities were accompanied by higher SOD activity with consequent rise in the production of $H_2O_2$. The formed hydrogen peroxide should be quickly eliminated via the activation of GSH-Px and CAT.

This explains why there was a decrease in the concentration of reduced GSH accompanied with a depletion of GSH-Px and CAT activities in groups manipulated with the examined compounds when it matched to control cells. As a result, the surplus of $H_2O_2$ formed in cancer cells treated with the compounds cannot be eliminated, thus we can say that the piling up of free radicals and $H_2O_2$ within cancer cells could be the cause of tumor cell death, at least partly [30].

Moreover, handling of both A549 and MCF-7 cells with compounds 1, 2, 3 and 4 led to considerable elevation in the concentration of NO. There is an increased indication demonstrating that NO is capable of activating programmed cell death by serving to make the membrane of mitochondria more permeable through dispelling the potential of the mitochondrial membrane [31]. Moreover, within the nucleus, nitric oxide has been shown to inhibit DNA repair enzymes [32], to cause gene mutation [33] and to initiate DNA strand breaks [34, 35], mentioned that, nearly all chemotherapeutic mediators encourage cells to excess production of ROS and therefore are able to motivate programmed cell death, and producing oxidative injury to DNA and proteins. Previously,[36] stated that controlling of substances which have

the ability to produce free radical may also have essential medical applications. These mechanisms by which ROS achieves the generating anticancer effect were only beginning to be explained. The pathways through which most anticancer agents induce its effect were believed to be a result of direct interference with DNA and obstruction of DNA regulatory machinery and the initiation of DNA damage through manufacturing of ROS [37].

**TABLE 1: Effect of treatment with the prepared compounds on the levels of reduced glutathione (GSH), hydrogen peroxide ($H_2O_2$) and nitric oxide (NO) in MCF-7 treated cells.**

| Compounds | GSH nmol/mg protein | $H_2O_2$ nmol/mg protein | NO µmol/mg protein |
|---|---|---|---|
| Control (DMSO) | 48.23±5.30 | 18.85±2.33 | 2.11 ± 0.31 |
| Doxorubicin | 16.85±1.90 [a] | 39.80±4.71 [a] | 4.81 ± 0.58[a] |
| 1 | 33.00±4.12[a] | 21.90±3.60[a,b] | 3.60 ± 0.47[a,b] |
| 2 | 20.70±0.31[a,b] | 37.70±4.10 [b] | 4.65 ± 0.50[a] |
| 3 | 25.20±3.11[b] | 28.90±2.50[a,b] | 4.00 ± 0.35[a,b] |
| 4 | 28.50±3.60[a] | 28.10±4.35[a,b] | 3.82 ± 0.44[a] |

Data are expressed as means ± S.E. of three separate experiments. [a] and [b] is significant difference from control and doxorubicin groups respectively at ($p < 0.05$).

**TABLE 2: Effect of treatment with the prepared compounds on the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) in MCF-7 treated cells.**

| Compounds | SOD U/mg Protein | CAT U/mg protein | GSH-Px U/mg protein |
|---|---|---|---|
| Control (DMSO) | 47.00±5.25 | 8.11±0.90 | 10.60±1.22 |
| Doxorubicin | 160.17±18.73 [a] | 2.10±0.26 [a] | 4.20±0.35 [a] |
| 1 | 77.00±6.30[a,b] | 4.22±0.38 | 7.70±0.88 [b] |
| 2 | 124.44±14.10[a] | 2.36±0.31[a] | 5.35±0.60[a] |
| 3 | 90.12±9.45 | 2.86±0.36[a] | 6.11±0.65[a] |
| 4 | 112.87±14.30[a] | 3.10±0.33[a] | 6.50±0.74[a] |

Data are expressed as means ± S.E. of three separate experiments. [a] and [b] is significant difference from control and doxorubicin groups respectively at ($p < 0.05$).

**TABLE 3: Effect of treatment with the prepared compounds on the levels of reduced glutathione (GSH), hydrogen peroxide ($H_2O_2$) and nitric oxide (NO) in A549 treated cells.**

| Compounds | GSH nmol/mg protein | $H_2O_2$ nmol/mg protein | NO (µmol/mg protein) |
|---|---|---|---|
| Control (DMSO) | 40.11±4.76 | 16.80±1.90 | 3.75 ± 0.48 |
| Doxorubicin | 19.72±2.07 [a] | 43.75±5.66 [a] | 6.30 ± 0.74[a] |
| 1 | 30.15±3.19 [a,b] | 22.30±2.66 [a,b] | 5.27 ± 0.66 [a,b] |
| 2 | 23.47±2.81[a] | 38.11±3.28[a] | 6.11 ± 0.72[a] |
| 3 | 26.18±3.21 [a,b] | 32.17±4.80 [a,b] | 5.65 ± 0.67 [a,b] |
| 4 | 28.26±4.12[a] | 28.20±3.17[a,b] | 5.60 ± 0.70 [a] |

Data are expressed as means ± S.E. of three separate experiments. [a] and [b] is significant difference from control and doxorubicin groups respectively at ($p < 0.05$).

**TABLE 4: Effect of treatment with the prepared compounds on the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) A549 treated cells.**

| Compounds | SOD U/mg protein | CAT U/mg protein | GSH-Px U/mg protein |
|---|---|---|---|
| Control (DMSO) | 41.30±4.75 | 7.81±0.83 | 9.60±1.45 |
| Doxorubicin | 180.60±20.70 [a] | 2.61±0.33 [a] | 5.13±0.63 [a] |
| 1 | 90.00±1.11[a,b] | 4.64±0.49 [a,b] | 7.80±0.91 [a,b] |
| 2 | 161.70±18.13[a,b] | 3.00±0.38[a,b] | 6.80±0.75 [a] |
| 3 | 138.80±15.11[a,b] | 3.65±0.41 [a,b] | 7.11±0.85[a] |
| 4 | 130.65±15.46[a,b] | 4.12±0.47 [a] | 7.50±0.82[a] |

Data are expressed as means ± S.E. of three separate experiments. [a] and [b] is significant difference from control and doxorubicin groups respectively at ($p < 0.05$).

In brief, the existing study proposed that the prepared compounds (**1-4**) have a noteworthy anticancer effect when it matched up to the result of the generally used anticancer treatment, doxorubicin, it produces their anticancer actions by adjusting free radical production through increasing SOD activity and decrease of catalase and glutathione peroxidase activities as well as depletion level of reduced glutathione accompanied with excess production of hydrogen peroxidase, nitric oxide and other free radicals leading to cancer cells mortem.

## Conclusion

The results demonstrate that the anticancer effect of the tested compounds 1, 2, 3 and 4 against human (MCF-7) breast cancer and human (A549) lung cancer cell lines may be stimulated via adjusting free radicals output. By raising the action of superoxide dismutase and dropping of intracellular glutathione concentration, catalase, glutathione peroxidase activities, come with excess production of hydrogen peroxide, nitric oxide and other free radicals leading to the death of cancer cells. The evaluation of the biological information obtained from our experiment indicated that the tested compounds could put forth a promising frame that may start the detection of more effective anticancer drugs. However, there is a great concerning about the selective creation of oxidative stress in cancer cells only to trigger cancer cell apoptosis and this aim initiates the need for further research.

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