SYNTHESIS, CHARACTERIZATION AND BIOLOGICAL EVALUATION OF NEW FUSED TRIAZINE DERIVATIVES BASED ON 6-METHYL-3-THIOXO-1,2,4-TRIAZIN-5-ONE

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Abstract: A one-pot reaction of 6-methyl-3-thioxo-3,4-dihydro-[1,2,4]triazin-5-one **1** with selected aldehydes **2a-d** and chloroacetic acid afforded the respective 2-arylidene-6-methyl-thiazolo[3,2-*b*][1,2,4]triazine-3,7-diones **4a-d**. Compunds **4a-d** could be also obtained *via* the reaction of **1** with chloroacetic acid in refluxing acetic acid to give 6-methyl-thiazolo[3,2-*b*][1,2,4]triazine-3,7-dione **3** then, Knoevenagel condensation of **3** with aldehydes **2a-d** gave compounds **4a-d**. Heterocyclization of **4a-c** with hydrazine hydrate and phenylhydrazine gave the corresponding pyrazolines **5a-c** and **6a-c**, respectively. Moreover, 7-amino-9-(aryl)-3-methyl-2-oxo-2*H*-pyrido[2',3':4,5][1,3]thiazolo[3,2-*b*][1,2,4]triazine-8-carbonitriles **7a-c** were synthesized by the reaction of **4a-c** with malononitrile in the presence of ammonium acetate. The structures of newly synthesized compounds were confirmed by analytical and spectroscopic measurements. Some selected new compounds were screened for their cytotoxic activities against three human cancer cell lines (HepG2, MCF-7 and A549) using SRB assay and the structure-activity relationship (SAR) was discussed. The biochemical assays including antioxidant enzyme, oxidative stress and estimation of nucleic acids and proteins have been discussed for some selected compounds. The molecular docking of **4c** and **7b** has been also studied.

Keywords: 1,2,4-triazin-5-one, thiazolo[3,2-*b*][1,2,4]triazine, fused triazine, structure-activity relationship, cytotoxicity, molecular docking

It is well know that 1,2,4-triazinone derivatives are very important class of heterocyclic compounds since they show a wide variety of applications both in the pharmaceutical and agrochemical industries. As potential human therapies, 1,2,4-triazinone derivatives have exhibited antimycobacterial (1), anticonvulsant (2) and antihemostatic effects (3). Moreover, fused triazine derivatives have significant biological activities as antimicrobial (4), antitumor (5, 6), anti-inflammatory and analgesic agents (7). In the agrochemical field, this class of compounds has shown activity as herbicides, for example Metribuzin and Goltix (8). Some commercially available veterinary drugs like Toltrazuril (Baycox®), Diclazuril (Vecoxan®) (9) and Ponazuril (Marquis®) (10) contain the triazinone moiety in their structures (Fig. 1).

In view of these facts and continuing our interest in synthesis of heterocyclic compounds with anticipated biological activities (11-13), we report herein the synthesis of some new series of fused triazine derivatives based on 6-methyl-3-thioxo-3,4-dihydro-[1,2,4]triazin-5-one and examination of their cytotoxicity and biochemical assays. The newly synthesized compounds were characterized by analytical and spectroscopic data (IR, MS and ^{1}H NMR).

MATERIALS AND METHODS

Chemistry

All melting points were measured on a Gallenkamp melting point apparatus and are uncorrected. The IR spectra were recorded (KBr discs) on a Perkin Elmer 1650 FT-IR instrument. The [1]H

Toltrazuril (Baycox®)
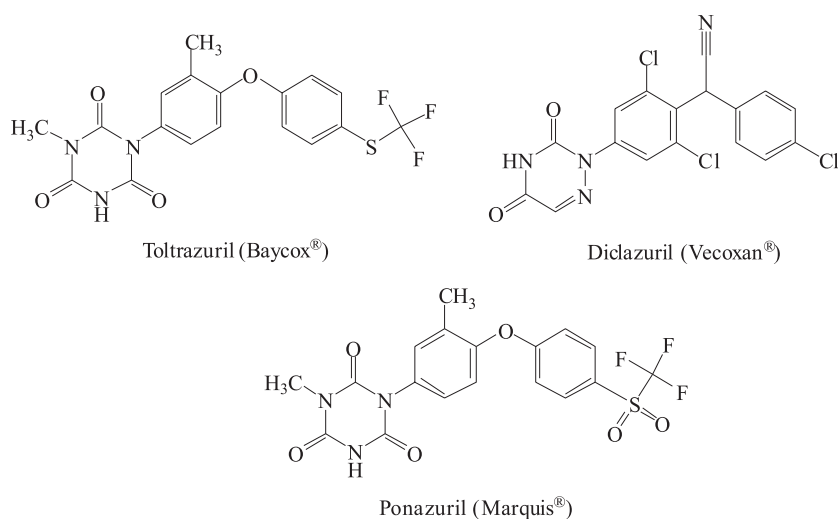
Diclazuril (Vecoxan®)

Ponazuril (Marquis®)

Figure 1. Structures of some drugs bearing the triazinone moiety

NMR (300 MHz) spectra were recorded on a Varian spectrometer using DMSO-d$_6$ as a solvent and TMS as an internal standard. Chemical shifts are reported in ppm. Mass spectra were recorded on a Varian MAT 112 spectrometer at 70 eV. Elemental analyses were carried out at The Micro Analytical Centre at Cairo University, Egypt.

Progress of the reactions was monitored by thin-layer chromatography (TLC) using aluminum sheets coated with silica gel F$_{254}$ (Merck) and viewing under a short-wavelength UV lamp effected detection.

All chemicals and reagents are of analytical grade. Dimethyl sulfoxide (DMSO), cisplatin and sulforhodamine-B stain (SRB) were purchased from Merck (Darmstadt, Germany). The other chemicals and reagents were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

6-Methyl-3-thioxo-3,4-dihydro-[1,2,4]triazin-5-one (**1**) (14) and 6-methyl-thiazolo[3,2-*b*][1,2,4]triazine-3,7-dione (**3**) (15) were prepared according to the literature procedures.

Synthesis of 2-arylidene-6-methyl-thiazolo[3,2-*b*][1,2,4]triazine-3,7-diones (4a-d)

General procedures

Procedure A: A mixture of 6-methyl-3-thioxo-3,4-dihydro-[1,2,4]triazin-5-one **1** (0.01 mol) with selected aldehydes **2a**-**d** [vanillin **2a**, 2,5-dimethoxybenzaldehyde **2b**, salicylaldehyde **2c** and/or ferrocene-2-carboxaldehyde **2d**] (0.01 mol) and chloroacetic acid (0.01 mol) in the presence of sodium acetate (0.01 mol) was refluxed in acetic

acid (40 mL) for 3 h. The reaction mixture was poured onto ice water (100 mL). The formed solid product was collected by filtration, dried and recrystallized from a suitable solvent.

Procedure B: A mixture of compound **1** (0.01 mol) and chloroacetic acid (0.01 mol) in the presence of sodium acetate (0.01 mol) was refluxed in acetic acid (40 mL) for 3 h to afford 6-methyl-thiazolo[3,2-*b*][1,2,4]triazine-3,7-dione **3**. Afterwards, compound **3** was reacted with selected aldehydes **2a**-**d** (0.01 mol) and sodium acetate (0.01 mol) in acetic acid (40 mL) for 3 h under reflux. The reaction mixture was poured onto ice water (100 mL). The formed solid product was collected by filtration, dried and recrystallized from a suitable solvent.

2-(4-Hydroxy-3-methoxybenzylidene)-6-methyl-thiazolo[3,2-*b*][1,2,4]triazine-3,7-dione (4a)

Yield: 69%, yellow crystals (dioxane), m.p. > 300°C. IR (KBr, cm^{-1}): 3257 (OH, br), 1727, 1672 (2C=O), 1563 (C=N). ^{1}H NMR (DMSO-d$_6$, δ, ppm): 2.28 (s, 3H, CH$_3$), 3.86 (s, 3H, OCH$_3$), 7.01 (d, 1H, J = 8.1 Hz, arom.), 7.28 (d, 1H, J = 8.1 Hz, arom.), 7.35 (s, 1H, arom), 8.17 (s, 1H, CH olefinic), 10.35 (s, 1H, OH, exchangeable with D$_2$O). Analysis: calcd. for C$_{14}$H$_{11}$N$_3$O$_4$S (317.32): C, 52.99; H, 3.49; N, 13.24; S, 10.10%; found: C, 53.19; H, 3.45; N, 13.35; S, 10.05%.

2-(2,5-Dimethoxybenzylidene)-6-methyl-thiazolo[3,2-*b*][1,2,4]triazine-3,7-dione (4b)

Yield: 65%, orange crystals (EtOH), m.p. 210-212°C. IR (KBr, cm^{-1}): 1745, 1678 (2C=O), 1593

(C=N). ^{1}H NMR (DMSO-d$_6$, δ, ppm): 2.30 (s, 3H, CH$_3$), 3.76, 3.79 (2s, 6H, 2OCH$_3$), 6.89-6.99 (m, 3H, arom.), 8.32 (s, 1H, CH olefinic). MS (m/z, % relative abundance): 331 (M$^+$, 22.00). Analysis: calcd. for C$_{15}$H$_{13}$N$_3$O$_4$S (331.35): C, 54.37; H, 3.95; N, 12.68; S, 9.68%; found: C, 54.50; H, 3.84; N, 12.80; S, 9.55%.

2-(2-Hydroxybenzylidene)-6-methyl-thiazolo[3,2-b][1,2,4]triazine-3,7-dione (4c)

Yield: 69%, white crystals (EtOH), m.p. 170-172°C. IR (KBr, cm^{-1}): 3396 (OH, br), 1689, 1662 (2C=O), 1599 (C=N). ^{1}H NMR (DMSO-d$_6$, δ ppm): 2.17 (s, 3H, CH$_3$), 7.35-7.79 (m, 4H, arom.), 8.16 (s, H, CH olefinic), 11.85 (s, 1H, OH exchangeable with D$_2$O). MS (m/z, % relative abundance): 289 (M$^+$+ 2H, 5.40). Analysis: calcd. for C$_{13}$H$_9$N$_3$O$_3$S (287.29): C, 54.35; H, 3.16; N, 14.63; S, 11.16%; found: C, 54.50; H, 3.05; N, 14.50; S, 11.00%.

2-(Ferrocen-2-ylidene)-6-methyl-thiazolo[3,2-b][1,2,4]triazine-3,7-dione (4d)

Yield: 72%, brown crystals (EtOH), m.p. > 300°C. IR (KBr, cm^{-1}): 1730, 1672 (2C=O), 1590 (C=N); ^{1}H NMR (DMSO-d$_6$, δ, ppm): 2.30 (s, 3H, CH$_3$), 4.20 (s, 5H, ferrocene ring protons), 4.61 (s, 2H, ferrocene ring protons), 4.80 (s, 2H, ferrocene ring protons), 8.10 (s, 1H, CH olefinic). Analysis: calcd. for C$_{17}$H$_{13}$FeN$_3$O$_2$S (379.21): C, 53.84; H, 3.46; N, 11.08; S, 8.46%; found: C, 54.00; H, 3.38; N, 11.00; S, 8.55%.

3-(Aryl)-7-methyl-2H-3,3a-dihydro-pyrazolo[3',4':4,5]thiazolo[3,2-b][1,2,4]triazin-6-ones (5a-c)
General procedure

A mixture of compounds 4a-c (0.01 mol) and hydrazine hydrate (0.01 mol) in ethanol (30 mL) containing 2-3 drops of glacial acetic acid was refluxed for 4-6 h. After cooling, the reaction mixture was poured onto ice water and the resulting solid was collected by filtration and recrystallized from ethanol to give compounds 5a-c.

3-(4-Hydroxy-3-methoxyphenyl)-7-methyl-2H-3,3a-dihydro-pyrazolo[3',4':4,5]thiazolo[3,2-b][1,2,4]triazin-6-one (5a)

Yield: 69%, reddish brown crystals, m.p. > 300°C. IR (KBr, cm^{-1}) 3332 (OH, br), 3223 (NH), 1679 (C=O), 1591 (C=N). Analysis: calcd. for C$_{14}$H$_{13}$N$_5$O$_3$S (331.35): C, 50.75; H, 3.95; N, 21.14; S, 9.68%; found: C, 50.50; H, 4.00; N, 21.00; S, 9.55%.

3-(2,5-Dimethoxyphenyl)-7-methyl-2H-3,3a-dihydro-pyrazolo[3',4':4,5]thiazolo[3,2-b][1,2,4]triazin-6-one (5b)

Yield: 70%, brown crystals, m.p. 190-192°C. IR (KBr, cm^{-1}): 3237 (NH), 1680 (C=O), 1595 (C=N); ^{1}H NMR (DMSO-d$_6$, δ, ppm): 2.33 (s, 3H, CH$_3$), 3.33 (d, 1H, CH-pyrazoline), 3.79, 3.82 (2s, 6H, 2OCH$_3$), 4.05 (d, 1H, CH-pyrazoline), 7.02-7.28 (m, 3H, arom.), 7.86 (s, br, 1H, NH, exchangeable with D$_2$O). Analysis: calcd. for C$_{15}$H$_{15}$N$_5$O$_3$S (345.38): C, 52.16; H, 4.38; N, 20.28; S, 9.28%; found: C, 52.00; H, 4.50; N, 20.15; S, 9.40%.

3-(2-Hydroxyphenyl)-7-methyl-2H-3,3a-dihydro-pyrazolo[3',4':4,5]thiazolo[3,2-b][1,2,4]triazin-6-one (5c)

Yield: 65%, brown crystals, m.p. 189-191°C. IR (KBr, cm^{-1}): 3417 (OH), 3225 (NH), 1698 (C=O), 1620 (C=N). ^{1}H NMR (DMSO-d$_6$, δ, ppm): 2.33 (s, 3H, CH$_3$), 3.15 (d, 1H, CH-pyrazoline), 3.93 (d, 1H, CH-pyrazoline), 7.00-7.77 (m, 4H, arom.), 7.87 (s, br, 1H, NH, exchangeable with D$_2$O), 10.15 (s, br, 1H, OH, exchangeable with D$_2$O). Analysis: calcd. for C$_{13}$H$_{11}$N$_5$O$_2$S (301.32): C, 51.82; H, 3.68; N, 23.24; S, 10.64%; found: C, 52.00; H, 3.58; N, 23.15; S, 10.50%.

3-(Aryl)-7-methyl-2-phenyl-3,3a-dihydro-pyrazolo[3',4':4,5]thiazolo[3,2-b][1,2,4]triazin-6-ones (6a-c)

General procedure

A mixture of compounds 4a-c (0.01 mol) and phenylhydrazine (0.01 mol) in ethanol (30 mL) containing 2-3 drops of glacial acetic acid was refluxed for 4-6 h. After cooling, the reaction mixture was poured onto ice water and the resulting solid was collected by filtration and recrystallized from ethanol to give 6a-c.

3-(4-Hydroxy-3-methoxyphenyl)-7-methyl-2-phenyl-3,3a-dihydropyrazolo[3',4':4,5]thiazolo[3,2-b][1,2,4]triazin-6-one (6a)

Yield: 65%, brown crystals, m.p. > 300°C. IR (KBr, cm^{-1}): 3403 (OH), 1653 (C=O); ^{1}H NMR (DMSO-d$_6$, δ, ppm): 2.34 (s, 3H, CH$_3$), 3.41 (d, 1H, CH-pyrazoline), 3.89 (s, 3H, OCH$_3$), 4.29 (d, 1H, CH-pyrazoline), 6.55-7.81 (m, 8H, arom.), 10.12 (s, br, 1H, OH, exchangeable with D$_2$O). Analysis: calcd. for C$_{20}$H$_{17}$N$_5$O$_3$S (407.45): C, 58.96; H, 4.21; N, 17.19; S, 7.87%; found: C, 59.16; H, 4.10; N, 17.25; S, 8.00%.

3-(2,5-Dimethoxyphenyl)-7-methyl-2-phenyl-3,3a-dihydropyrazolo[3',4':4,5]thiazolo[3,2-b][1,2,4]triazin-6-one (6b)

Yield: 75%, yellowish brown crystals, m.p. 183-184°C. IR (KBr, cm⁻¹): 1686 (C=O), 1592 (C=N). ¹H NMR (DMSO-d_6, δ, ppm): 2.35 (s, 3H, CH₃), 3.81 (d, 1H, CH-pyrazoline), 3.75, 3.80 (2s, 6H, 2OCH₃), 4.00 (d, 1H, CH-pyrazoline), 6.91-7.31 (m, 8H, arom.); MS (*m/z*, % relative abundance): 421 (M⁺, 3.20). Analysis: calcd. for $C_{21}H_{19}N_5O_3S$ (421.47): C, 59.84; H, 4.54; N, 16.62; S, 7.61%; found: C, 60.10; H, 4.50; N, 16.55; S, 7.50%.

3-(2-Hydroxyphenyl)-7-methyl-2-phenyl-3,3a-dihydropyrazolo[3',4':4,5]thiazolo[3,2-*b*][1,2,4]triazin-6-one (6c)

Yield: 65%, brown crystals; m.p. 186-188°C, IR (KBr, cm⁻¹): 3444 (OH), 1668 (C=O), 1600 (C=N). ¹H NMR (DMSO-d_6, δ, ppm): 2.30 (s, 3H, CH₃), 3.75 (d, 1H, CH-pyrazoline), 4.11 (d, 1H, CH-pyrazoline), 6.79-7.33 (m, 9H, arom.), 10.18 (s, *br*, 1H, OH, exchangeable with D₂O). Analysis: calcd. for $C_{19}H_{15}N_5O_2S$ (377.42): C, 60.46; H, 4.01; N, 18.56; S, 8.50%; found: C, 60.60; H, 3.90; N, 18.40; S, 8.40%.

7-Amino-9-(aryl)-3-methyl-2-oxo-2*H*-pyrido[2',3':4,5][1,3]thiazolo[3,2-*b*][1,2,4]triazine-8-carbonitriles (7a-c)

To a solution of **4a-c** (0.01 mol) in absolute ethanol (30 mL) containing ammonium acetate (0.01 mol), malononitrile (0.01 mol) was added. The reaction mixture was refluxed for 10 h. After cooling, the reaction mixture was poured onto ice water mixture containing a few drops of hydrochloric acid. The resulting solid was collected by filtration and recrystallized from ethanol to give **7a-c**

7-Amino-9-(4-hydroxy-3-methoxyphenyl)-3-methyl-2-oxo-2*H*-pyrido[2',3':4,5][1,3]thiazolo[3,2-*b*][1,2,4]triazine-8-carbonitrile (7a)

Yield: 59%, brown crystals, m.p. > 300°C. IR (KBr, cm⁻¹): 3440 (OH), 3401 (NH₂), 2221 (C≡N), 1643 (C=O). ¹H NMR (DMSO-d_6, δ, ppm): 2.37 (s, 3H, CH₃), 3.82 (s, 3H, OCH₃), 6.98-7.14 (m, 3H, arom.), 9.30 (s, 2H, NH₂, exchangeable with D₂O), 9.80 (s, br, 1H, OH, exchangeable with D₂O). Analysis: calcd. for $C_{17}H_{12}N_6O_3S$ (380.38): C, 53.68; H, 3.18; N, 22.09; S, 8.43%; found: C, 53.50; H, 3.25; N, 22.00; S, 8.35%.

7-Amino-9-(2,5-dimethoxyphenyl)-3-methyl-2-oxo-2*H*-pyrido[2',3':4,5][1,3]thiazolo[3,2-*b*][1,2,4]triazine-8-carbonitrile (7b)

Yield: 71%, brown crystals, m.p. 157-159°C. IR (KBr, cm⁻¹): 3360 (NH₂), 2211 (C≡N), 1662 (C=O). ¹H NMR (DMSO-d_6, δ, ppm): 2.37 (s, 3H,

CH₃), 3.76, 3.82 (2s, 6H, 2OCH₃), 6.90-7.18 (m, 3H, arom.), 9.31 (s, 2H, NH₂, exchangeable with D₂O). Analysis: calcd. for $C_{18}H_{14}N_6O_3S$ (394.41): C, 54.81; H, 3.58; N, 21.31; S, 8.13%; found: C, 55.00; H, 3.50; N, 21.21; S, 8.05%.

7-Amino-9-(2-hydroxyphenyl)-3-methyl-2-oxo-2*H*-pyrido[2',3':4,5][1,3]thiazolo[3,2-*b*][1,2,4]triazine-8-carbonitrile (7c)

Yield: 68%, brown crystals, m.p. 145-147°C. IR (KBr, cm⁻¹): 3421 (OH), 3328 (NH₂), 2203 (C≡N), 1658 (C=O). ¹H NMR (DMSO-d_6, δ, ppm): 2.30 (s, 3H, CH₃), 6.98-7.64 (m, 4H, arom.), 9.20 (s, 2H, NH₂, exchangeable with D₂O). 10.05 (s, br, 1H, OH, exchangeable with D₂O). Analysis: calcd. for $C_{16}H_{10}N_6O_2S$ (350.35): C, 54.85; H, 2.88; N, 23.99; S, 9.15%; found: C, 55.00; H, 2.80; N, 24.10; S, 9.10%.

Cytotoxicity
Cell lines and culturing

Cytotoxicity screening for the tested compounds was utilizing 3 different human tumor cell lines namely, breast cancer MCF-7, human liver cancer HepG2 and human lung cancer A549 which were obtained from the American Type Culture Collection (Rockville, MD, USA). The tumor cells were maintained 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°C in humidified atmosphere containing 5% CO₂. Cells at a concentration of 0.50×10^6 were grown in a 25 cm² flasks in 5 mL of complete culture medium.

Cytotoxicity assay

Cytotoxic activity was measured *in vitro* using the sulforhodamine-B stain (SRB) assay according to the previously reported standard procedure (16). Cells were inoculated in 96-well microtiter plate (10⁴ cells/well) for 24 h before treatment with the tested compounds to allow attachment of cell to the wall of the plate. Test compounds were dissolved in DMSO and diluted with saline solution to the appropriate volume. Different concentrations of the compounds under testing (0, 5, 10, 20, 40 μg/mL) were added to the cells. Triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the compounds for 48 h at 37°C and in an atmosphere of 5% CO₂. After 48 h, cells were fixed, washed and stained for 30 min with 0.4% (w/v) SRB dissolved in 1% acetic acid. The unbound dye was removed by four washes with 1% acetic acid and attached stain was recovered with Tris-EDTA

buffer. Color intensity was measured in an ELISA reader. The relation between surviving fraction and drug concentration is plotted to get the survival curve for each cell line after the specified time. The concentration required for 50% inhibition of cell viability (IC_{50}) was calculated and the results are given in Tables 1 and 2.

Biochemical assays

The cells in culture medium were treated with 20 µL of 1/10 of IC_{50} values of the compounds or the standard reference drug, cisplatin, then incubated for 24 h at 37°C, in a humidified 5% CO_2 atmosphere. The cells were harvested and homogenates were prepared in saline solution using a tight pestle

homogenizer until complete cell disruption for further biochemical analysis. The supernatants obtained after centrifugation of cell homogenates were used for determination of the following parameters.

Antioxidant enzyme assays

The activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) were determined according to reported methods (17-19).

Oxidative stress assays

The levels of hydrogen peroxide (H_2O_2), nitric oxide (NO) and reduced glutathione (GSH) were determined by known methods (20-22).

Table 1. Effect of treatment at various concentrations of tested compounds on human breast cancer cell line MCF-7, cytotoxicity (IC_{50}) as measured with SRB method.

Tested compounds	Concentration (µg/mL)	Cell viability (%)	IC_{50} (µg/mL)
Cisplatin	0 5 10 20 40	100 40 20 15 9	4.00
5b	0 5 10 20 40	100 80 55 33 11	12.20
6a	0 5 10 20 40	100 70 45 25 10	9.00
6b	0 5 10 20 40	100 58 40 20 9	7.00
6c	0 5 10 20 40	100 55 45 24 7	6.80
7a	0 5 10 20 40	100 60 30 14 9	6.60
7b	0 5 10 20 40	100 50 35 21 11	5.00

Table 2. Effect of treatment at various concentrations of tested compounds on human liver cancer cell line HepG2, cytotoxicity (IC_{50}) as measured with SRB method.

Tested compounds	Concentration (µg/mL)	Cell viability (%)	IC_{50} (µg/mL)
Cisplatin	0 5 10 20 40	100 30 25 15 8	3.50
4a	0 5 10 20 40	100 55 27 16 9	6.00
4b	0 5 10 20 40	100 50 24 11 7	5.00
4c	0 5 10 20 40	100 45 35 20 11	4.60
4d	0 5 10 20 40	100 80 50 11 3	10.00

Estimation of nucleic acids and protein

Nucleic acids (DNA and RNA) and total protein were precipitated and measured in cell homogenates. Total DNA was extracted and assayed according to the reported method (23), total RNA was extracted and assayed according to the method provided by Hybaid/AGS (Germany) and total cellular protein was assayed according to a reported method (24).

Statistical analysis

The results are reported as the mean ± standard error (S.E.) for at least three experiments. Statistical differences were analyzed according to one way ANOVA test followed by Student's t test wherein the differences were considered to be significant at $p < 0.05$.

Molecular docking study

The molecular docking is performed and analyzed with the MOE program. Inosine-5'-monophosphate dehydrogenase (IMPDH) is an essential cytoplasmic purine metabolic enzyme that catalyzes the NAD-dependent oxidation of inosine monophosphate (IMP) to xanthosine monophosphate (XMP). IMPDH expression is found to be upregulated in tumor tissues and tumor cell lines (25, 26). Accordingly, human IMPDH is a target of clinical anti-cancer drugs.

The synthesized compounds **4c** and **7b** are investigated for the binding affinity of inosine-5'-monophosphate dehydrogenase receptor (pdb:1NFB). Its purpose is of lead optimization and to find out the interaction between compounds **4c, 7b** and the inosine-5'-monophosphate dehydrogenase (IMPDH) receptor.

Molecular modeling calculations and local docking were done by using MOE (molecular modeling environment) to evaluate the binding free energies of these inhibitors into the target IMPDH receptor.
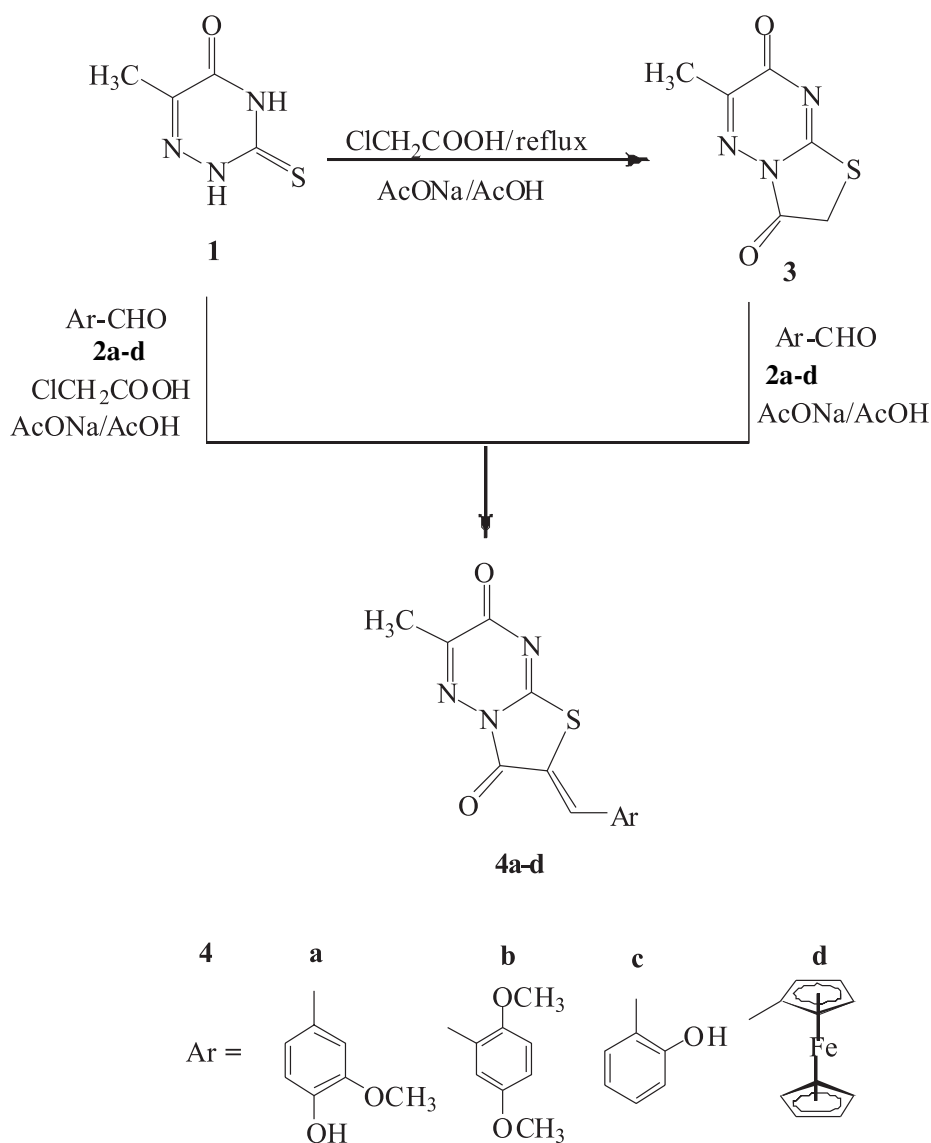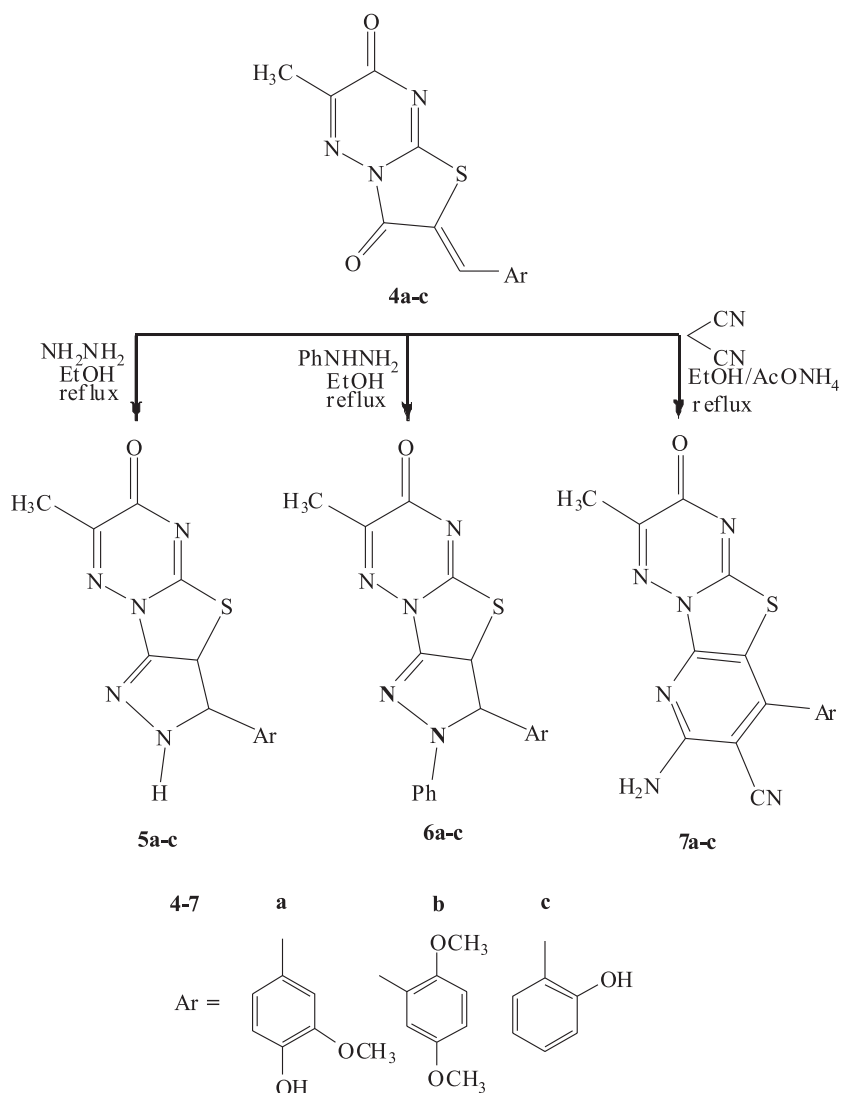
RESULTS AND DISCUSSION

Chemistry

The starting compounds, 6-methyl-3-thioxo-3,4-dihydro-1,2,4-triazin-5-one (**1**) (14) and 6-methyl-thiazolo[3,2-*b*][1,2,4]triazine-3,7-dione (**3**) (15) were utilized in preparing the target compounds (Schemes 1 and 2).

It has been now found that a one-pot reaction of **1** with some selected aldehydes **2a-d** [namely, vanillin **2a**, 2,5-dimethoxy benzaldehyde **2b**, salicyl-aldehyde **2c** and ferrocene-2-carboxaldehyde **2d**] and chloroacetic acid in the presence of sodium acetate in refluxing acetic acid gave the respective new 2-arylidene-6-methyl-thiazolo[3,2-*b*][1,2,4]tri-azine-3,7-dione derivatives **4a-d**. The same compounds could be also obtained upon reacting compound **1** with chloroacetic acid and sodium acetate in refluxing acetic acid to give **3**. Knoevenagel condensation (27) of compound **3** with aldehydes **2a-d** gave compounds **4a-d**, respectively (Scheme 1).

Structures of compounds **4a-d** were confirmed on the basis of their analytical and spectral data. For compound, 2-(4-hydroxy-3-methoxyben-zylidene)-6-methyl-thiazolo[3,2-*b*][1,2,4]triazine-3,7-dione (**4a**), taken as a representative example, its IR spectrum (KBr, cm⁻¹) showed a broad band at 3257 corresponding to -OH and two bands at 1727 and 1658 due to two C=O groups. Its ¹H NMR spectrum (DMSO-d₆, δ, ppm) showed two signals at 2.28 and 3.86 due to -CH₃ and -OCH₃ group protons, respectively. The aromatic protons (3H) appeared as a singlet at 7.35 and two doublets (each with J = 8.1 Hz) at 7.01 and 7.28 ppm due to

Scheme 1. Synthesis of compounds **4a-d**

Scheme 2. Synthesis of compounds **5a-c**, **6a-c** and **7a-c**

the AB system of the vanillin ring. The singlet that appeared at 8.17 ppm is attributed to the olefinic (C=C$\underline{H}$Ar) proton. Moreover, the signal present at 10.35 was assigned for -OH proton, which was D$_2$O exchangeable.

The newly synthesized arylidene derivatives **4a-d** have been now investigated as key molecules for building new fused heterocycles through the addition reaction on their exocyclic olefinic double bond with some selected nucleophiles, namely: hydrazine hydrate, phenylhydrazine and malononitrile to give compounds **5**, **6** and **7**, respectively (Scheme 2). The reactivity of the olefinic bond towards nucleophilic attack may be enhanced through its conjugation with the carbonyl group.

Thus, cycloaddition reactions of 2-arylidene-6-methyl-thiazolo[3,2-*b*][1,2,4]triazine-3,7-dione **4a-c** with hydrazine hydrate and phenylhydrazine in ethanol afforded the 3-(aryl)-7-methyl-2*H*-3,3a-dihydro-pyrazolo[3',4':4,5]thiazolo[3,2-*b*][1,2,4] triazin-6-ones (**5a-c**) and 3-(aryl)-7-methyl-2-phenyl-3,3a-dihydro-pyrazolo[3',4':4,5]thiazolo[3,2-*b*][1,2,4]triazin-6-ones (**6a-c**), respectively (Scheme 2).

Structures of **5a-c** and **6a-c** were established due to their analytical and spectral data. Thus, as an example, the IR spectrum (KBr, cm^{-1}) of compound **5c** showed bands at 3417 for -OH, 3225 for -NH and 1698 for C=O. Its ^{1}H NMR spectrum (DMSO-d$_6$, δ, ppm) revealed the presence of two doublets at 3.15 and 3.93 ppm due to the two vicinal methine protons

of the pyrazoline ring. The two signals at 7.87 and 10.15 ppm are corresponding to -NH and -OH protons, respectively, which were D_2O exchangeable. The aromatic protons (4H) appeared as a multiplet in the 7.00-7.77 region.

Moreover, the reaction of compounds **4a-c** with malononitrile in refluxing absolute ethanol in the presence of ammonium acetate gave the corresponding 7-amino-9-(aryl)-3-methyl-2-oxo-2*H*-pyrido[2',3':4,5][1,3]thiazolo[3,2-*b*][1,2,4]triazine-8-carbonitriles **7a-c** as brown crystals (Scheme 2).

Structures of compounds **7a-c** were confirmed on the basis of elemental analysis and spectral data. As an example, the IR spectrum (KBr, cm^{-1}) of 7-amino-9-(4-hydroxy-3-methoxyphenyl)-3-methyl-2-oxo-2*H*-pyrido[2',3':4,5][1,3]thiazolo[3,2-*b*][1,2,4]triazine-8-carbonitrile **7a** showed strong stretching absorption bands at 3440 and 3401 for -OH and -NH$_2$, respectively, and a band at 2221 for C≡N group. Its ^{1}H NMR spectrum (DMSO-d$_6$, δ, ppm) showed two signals at 9.30 and 9.80 ppm due to -NH$_2$ and -OH protons, respectively, which were D$_2$O exchangeable, the singlet that appeared at 3.82 ppm is attributed to protons of the OCH$_3$ group while the aromatic protons (3H) appeared as a multiplet in the 6.98-7.14 ppm region.

Cytotoxicity

Cytotoxicity of 13 tested compounds was evaluated against human breast cancer cell line MCF-7, human liver cancer cell line HepG2 and human lung cancer cell line A549 using sulforhodamine B (SRB) colorimetric assay, in comparison with cisplatin as a reference drug. Cytotoxic activity is expressed as median growth inhibitory concentration (IC$_{50}$) and has been compiled in Tables 1 and 2. From these results, it is evident that six of the investigated compounds have displayed a potent growth inhibitory activity against human breast cancer cell line MCF-7 (Table 1), while four compounds have displayed a potent growth inhibitory activity against HepG2 (Table 2). On the other hand, none of the investigated compounds has exerted any activity against human lung cancer cell line A549.

Both of MCF-7 and HepG2 cell lines showed normal growth in the culture system. Meanwhile, DMSO did not exert any noticeable effect on cellular growth. It was also observed that there was a gradual decrease in the viability of cancer cells with increasing the concentration of the tested compounds in a dose-dependent inhibitory effect.

Against MCF7 cell line, the potency of the investigated compounds decreased in the order **7b** > **7a** > **6c** > **6b** > **6a** > **5b**. Compound **7b** was found to

Table 3. Effect of treatment with the tested compounds on the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), as well as the levels of reduced glutathione (GSH) and hydrogen peroxide (H$_2$O$_2$) in MCF-7 and HepG2 treated cells.

Cell lines	Tested compounds	SOD U/mg protein	CAT U/mg protein	GSH-Px U/mg protein	GSH nmol/mg protein	H$_2$O$_2$ nmol/mg protein
MCF-7	Control (DMSO)	40.00 ± 3.90	7.90 ± 0.80	10.20 ± 1.00	45.00 ± 4.80	18.80 ± 1.90
	Cisplatin	140.0 ± 14.20[a,b]	3.00 ± 0.26[a]	5.00 ± 0.47[a]	17.50 ± 1.80[a]	70.50 ± 7.60[a]
	5b	70.00 ± 6.80[a,b]	6.00 ± 0.60[a]	8.80 ± 0.90[a]	40.50 ± 6.30[a]	32.80 ± 3.40[a,b]
	6a	80.50 ± 8.00[a,b]	5.40 ± 0.60[a]	7.85 ± 0.70[a]	33.80 ± 0.38[a,b]	36.50 ± 3.20[a,b]
	6b	85.20 ± 9.00[a,b]	5.00 ± 0.52[a]	7.50 ± 0.76[a]	28.50 ± 3.00[a,b]	40.50 ± 5.00[a,b]
	6c	90.20 ± 8.60[a,b]	4.40 ± 0.50[a]	7.20 ± 0.70[a]	26.50 ± 2.30[a,b]	55.30 ± 5.70[a,b]
	7a	100.0 ± 11.00[a,b]	3.90 ± 0.39[a]	6.70 ± 0.65[a]	22.80 ± 2.20[a]	60.00 ± 5.80[a]
	7b	120.00 ± 11.70[a,b]	3.70 ± 0.24[a]	6.20 ± 0.30[a]	19.00 ± 1.90[a]	65.30 ± 6.00[a]
HepG2	Control (DMSO)	35.20 ± 3.60	7.75 ± 0.80	9.50 ± 0.90	45.00 ± 4.70	15.00 ± 1.50
	Cisplatin	140.00 ± 15.50[a]	3.00 ± 0.30[a]	4.50 ± 0.40[a]	20.00 ± 2.20[a]	50.00 ± 4.80[a]
	4a	100.00 ± 12.20[a,b]	4.30 ± 0.46[a,b]	6.90 ± 0.78[a]	33.80 ± 3.80[a,b]	35.20 ± 3.80[a]
	4b	112.60 ± 7.90[a,b]	4.00 ± 0.50[b]	6.60 ± 0.80[a,b]	29.50 ± 3.00[a,b]	41.20 ± 4.00[a,b]
	4c	135.60 ± 8.20[a,b]	3.60 ± 0.42[a,b]	5.30 ± 0.60[a,b]	27.50 ± 2.50[a,b]	45.80 ± 4.60[a,b]
	4d	90.60 ± 8.70[a,b]	4.30 ± 0.46[a,b]	7.00 ± 0.70[a]	37.50 ± 4.20[a,b]	29.00 ± 3.00[a,b]

Data are expressed as the means ± S.E. of three separate experiments. [a] and [b] are significant differences from control and cisplatin groups, respectively, at p < 0.05.

Table 4. Effect of the tested compounds on the level of total protein, nucleic acids (RNA and DNA) and nitric oxide (NO) in MCF-7 and HepG2 treated cells.

Cell lines	Tested compounds	Protein ($\mu g/10^6$ cells)	RNA ($\mu g/10^6$ cells)	DNA ($\mu g/10^6$ cells)	NO ($\mu mol/mg$ protein)
MCF-7	Control (DMSO)	150.50 ± 14.80	22.30 ± 2.00	15.00 ± 1.50	3.00 ± 0.29
	Cisplatin	50.30 ± 4.85[a]	5.20 ± 0.50[a]	4.00 ± 0.38[a]	8.00 ± 0.75[a]
	5b	100.60 ± 9.80[a]	15.30 ± 0.58[a,b]	11.30 ± 1.26[a,b]	4.00 ± 0.40[a]
	6a	85.60 ± 8.60[a,b]	13.50 ± 0.90[a,b]	9.00 ± 0.85[a,b]	4.80 ± 0.48[a,b]
	6b	80.60 ± 8.10[a,b]	11.00 ± 0.76[a,b]	7.90 ± 0.80[a,b]	5.80 ± 0.60[a,b]
	6c	77.00 ± 7.50[a,b]	9.00 ± 0.85[a,b]	7.30 ± 0.80[a,b]	6.20 ± 0.56[a,b]
	7a	67.60 ± 4.00[a]	7.70 ± 0.36[a]	7.00 ± 0.65[a,b]	7.00 ± 0.70[a]
	7b	60.00 ± 6.00[a]	6.80 ± 0.66[a]	5.70 ± 0.60[a]	7.70 ± 0.80[a]
HepG2	Control (DMSO)	125.60 ± 12.00	20.70 ± 2.40	10.00 ± 0.90	2.00 ± 0.20
	Cisplatin	40.00 ± 4.20[a]	5.00 ± 0.45[a]	4.50 ± 0.50[a]	5.00 ± 0.50[a]
	4a	75.30 ± 8.20[a,b]	8.00 ± 0.84[a,b]	6.30 ± 0.60[a]	3.90 ± 0.33[a]
	4b	70.20 ± 7.20[a,b]	7.60 ± 0.70[a,b]	5.60 ± 0.60[a,b]	4.50 ± 0.42[a]
	4c	60.00 ± 6.30[a,b]	6.80 ± 0.80[a]	5.30 ± 0.60[a,b]	4.60 ± 0.40[a]
	4d	80.60 ± 7.70[a,b]	9.60 ± 0.90[a,b]	7.60 ± 0.80[a,b]	3.20 ± 0.30[a,b]

The values are expressed as the mean ± S.E. of three separate experiments. [a] and [b] are significant differences from control and cisplatin groups, respectively, at $p < 0.05$.

be the most potent (IC_{50}: 5.00 µg/mL) amongst the tested compounds and was almost as efficacious as the reference drug cisplatin (IC_{50}: 4.00 µg/mL) (Table 1). Moreover, the potency of compounds **4a-c** against HepG2 cell line decreased in the order **4c** > **4b** > **4a** > **4d** where they recorded IC_{50} (µg/mL) values of 4.60, 5.0, 6.0 and 10.0, respectively. Compound **4c** showed a potency (IC_{50}: 4.60 µg/mL) which is relatively near to that of the reference drug, cisplatin (IC_{50}: 3.50 µg/mL) (Table 2)

Based on these results, it is evident that there is a structure-activity relationship (SAR). As shown in Table 1 and 2, from the screening of the tested compounds against the MCF-7 and HepG2 cell lines, it was found that some derivatives linked by 2,5-dimethoxyphenyl group were more active than their respective analogues with 4-hydroxy-3-methoxy-phenyl group. Thus, compounds **4b** (IC_{50}: 5.00 µg/mL) and **7b** (IC_{50}: 5.00 µg/mL) were found to be more potent than **4a** (IC_{50}: 6.00 µg/mL) and **7a** (IC_{50}: 6.60 µg/mL), respectively.

Biochemical assays

To elucidate the mechanism by which the tested compounds exert their cytotoxic activities, these compounds were selected to estimate their ability to induce oxidative stress in the two human cancer cell lines (human breast cancer cell line MCF-7 and human liver cancer cell line HepG2). We estimated the activities of the free-radical-metabolizing enzymes including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) as well as the levels of the oxidative stress parameters including hydrogen peroxide (H_2O_2), nitric oxide (NO) and reduced glutathione (GSH) in cells treated with tested compounds. Moreover, the effect of these compounds on the levels of total protein and nucleic acids was estimated.

As shown in Table 3, general treatment of the cells with tested compounds or cisplatin (at the 1/10 of IC_{50} values) (28) resulted in a significant increase in the activity of SOD and the level of H_2O_2 to be higher than those of the control, accompanied with a significant decrease in the activity of CAT and GSH-Px, and depletion in GSH level, indicating an increase in the cellular levels of reactive oxygen species. This means that the cytotoxic activity of these compounds was accompanied with high activity of SOD with subsequent increase in H_2O_2 production. The produced H_2O_2 should be rapidly removed through the activation of CAT and GSH-Px. The present results showed that activities of CAT and GSH-Px and the level of reduced GSH

were lowered in case of the groups treated with the tested compounds compared to the control cells. Consequently, the excess H_2O_2 that produced in tumor cells with the tested compounds cannot be removed. In other words, the accumulation of H_2O_2 and other free radicals in tumor cells may be partially the cause of tumor cell killing. Changes in the activities of the free-radical-metabolizing enzymes and oxidative stress parameters of the tested compounds were found to be in the order: cisplatin > **7b** > **7a** > **6c** > **6b** > **6a** > **5b** for MCF-7 while for HepG2 cell line the order was: cisplatin > **4c** > **4b** > **4a** > **4d** which is in accordance with the order of cytotoxic activity of the tested compounds against both cell lines.

The highest activity was found for the most potent cytotoxic compound **7b** in MCF-7 and compound **4c** in HepG2 cell lines, which resulted in

the highest SOD activity and H_2O_2 and low activities of CAT and GSH-Px as well as GSH level than the other tested compounds. The consistency between cytotoxic activity and biochemical assay results indicates that the cytotoxic effect of the tested compounds may be exerted, at least partially, by production of ROS. Moreover, results in Table 4 indicate that treatment of both MCF-7 and HepG2 cells with these compounds led to a significant increase in the level of NO. These results were in accordance with the reported method (29) that the polysaccharides isolated from algae have been applied to modify the macrophage activity by inducing the production of cytokines and nitric oxide. There is a growing body of evidence indicating that NO is able to induce apoptosis by dissipating the membrane potential of mitochondria and to make it more permeable (30). In addition,
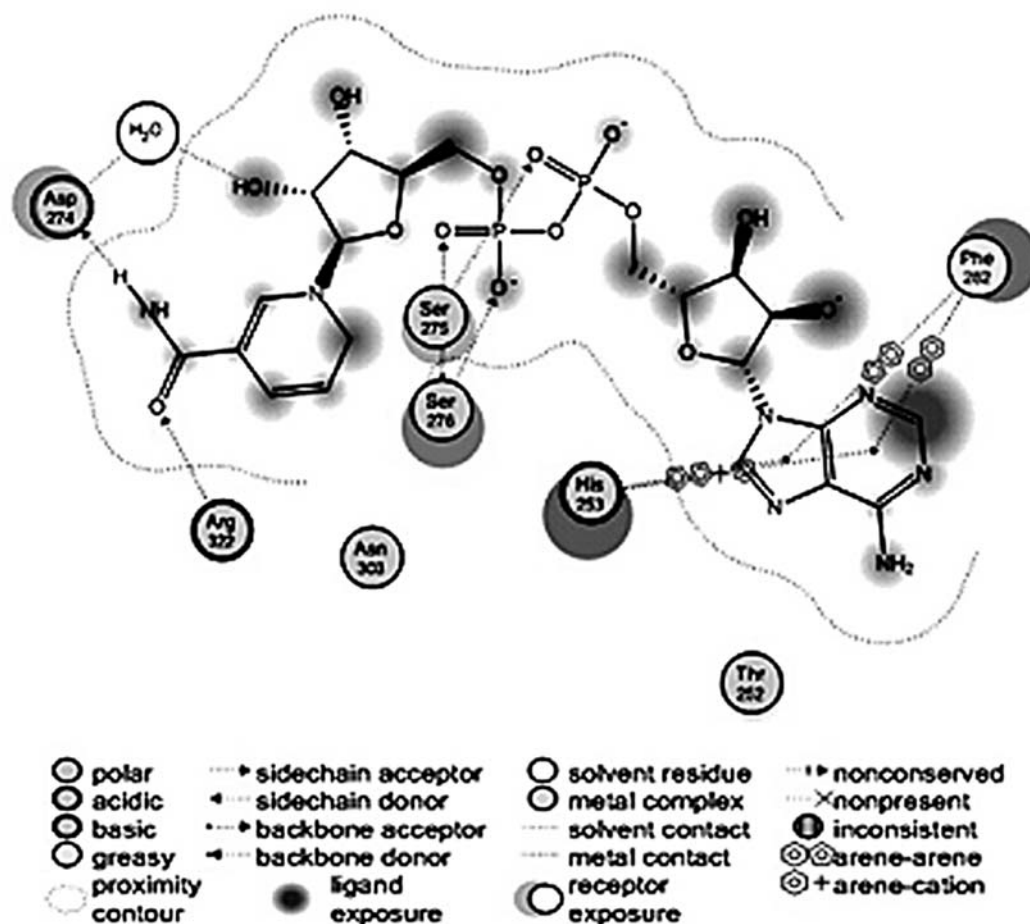

Figure 2. The ligand interaction and the binding mode of the native ligand nicotinamide-adenine-dinucleotide (NAD) it is exhibited one H-bond donor with ASP 274 and at distance 2.27 and one H-bond donor with HOH 731and at distance 2.74 and one H-bond acceptor with SER 275 at distance 2.45 and three H-bond acceptor with SER 276 at distance 2.77, 2.95, 2.70 and one H-bond acceptor with ARG 322 at distance 2.84 and one H-bond acceptor with HOH 731 at distance 2.74 shown as hatched line and it is score -22.314

the elevated level of NO was accompanied with a depletion in the levels of total protein and nucleic acids as compared to the control. This can be explained by several cytotoxic effects that include reaction of NO with proteins and nucleic acids. The main targets of NO in proteins are the thiol group (31) and iron of active sites (32) in the nucleus. NO has been found to cause gene mutation (33) to inhibit DNA repair enzymes (34) and to mediate DNA strand breaks (35). Moreover, it was reported that most chemotherapeutic agents cause cells to overgenerate ROS and thus, are capable of inducing apoptosis, and causing oxidative damage to DNA and proteins (36). The cascade of signals mediating apoptosis often involves a ROS intermediate messenger and ROS can short circuit the pathway, by passing the need for upstream signals for cell suicide. Latter, Huang et al. (37) reported that the regulation of free radical-producing agents may also have important clinical applications. This mechanism for the effects of ROS generating anticancer agents is only in the beginning to be understood, as previously the mechanism of most anticancer agents was believed to be mainly due to a direct interaction with DNA, an interference with DNA regulatory machinery (e.g., topoisomerases and helicases) and as well as due to the initiation of DNA damage *via* production of ROS (38).

Molecular docking study

The docking studies were carried out using Molecular Operating Environment (MOE) 2008.10 (Moe source: Chemical Computing Group Inc., Quebec, Canada, 2008). First of all, a Gaussian contact surface around the binding site is drawn. The surface surrounds the van der Waals surface of a molecule (filling in solvent inaccessible gaps). Then, docking studies are carried out to evaluate the binding free energy of the inhibitors within the macromolecules. The dock scoring in MOE software is done using London dG scoring function and has been enhanced by using two different refinement methods. The force-field and Grid-Min pose have been updated to ensure that refined poses satisfy the specified conformations. Rotatable bonds were allowed where the best 10 poses were retained and analyzed for the binding poses best score. The database browser was used in MOE to compare the docking poses to the ligand in the co-crystallized structure and to get RMSD of the docking pose compared to the co crystal ligand position.

Preparation of ligands and target inosine-5'-monophosphate dehydrogenase receptor

In this study, ligands **4c** and **7b** were studied for their binding affinity into PTK. The Molecule Builder tool in MOE was used to construct a three-dimensional model of their structures. Energy minimization
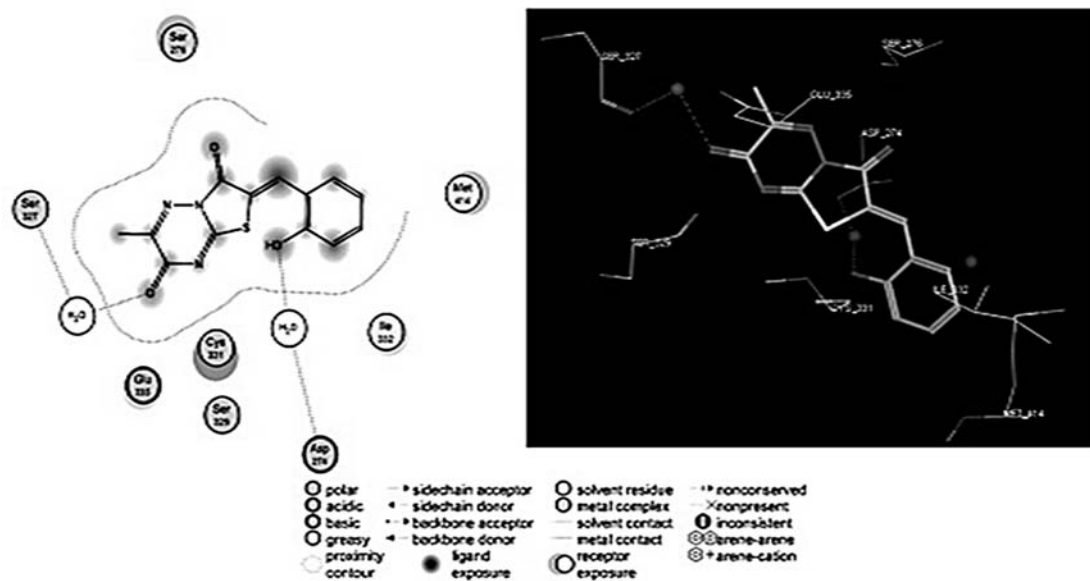

Figure 3. Ligand interaction and the binding mode of compound **4c** with receptor, exhibited one H-bond donor with HOH 731 at distance 2.95 and one H-bond acceptor with HOH731 at distance 2.95 and H-bond acceptor with HOH 742 at distance 2.56 shown as hatched line similar to co-crystallized ligand it is give score -13.01.
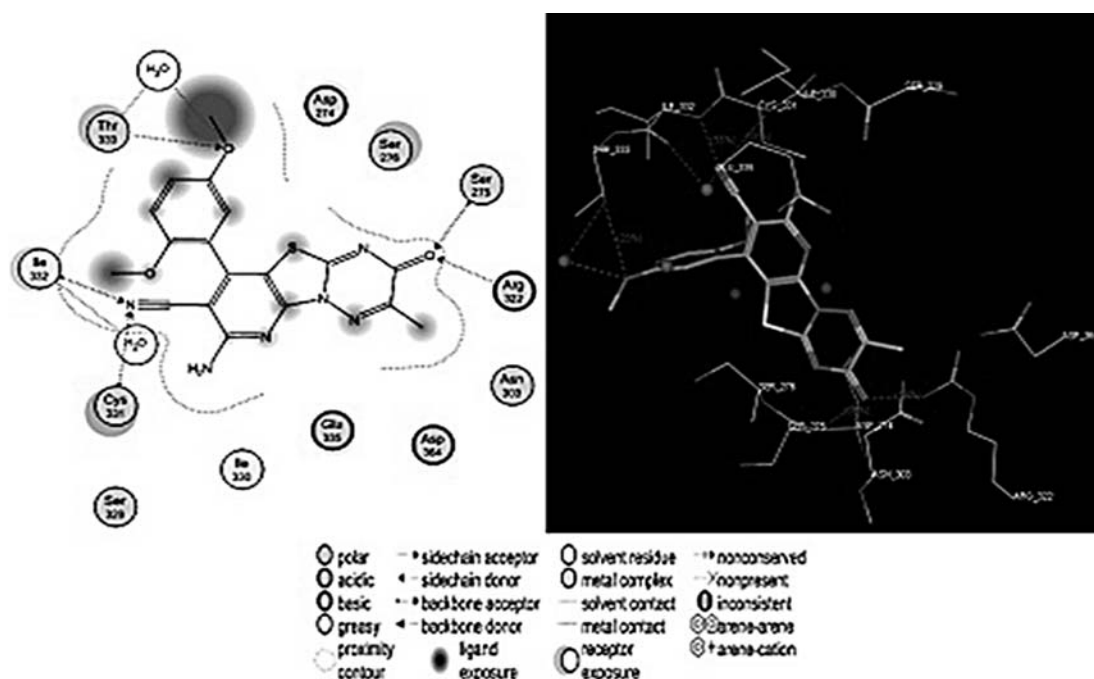
Figure 4. Ligand interaction and the binding mode of compound **7b** with Aurora kinase receptor, exhibited one H-bond acceptor with SER 275 at distance 2.49 and one H-bond acceptor with ARG 322 at distance 2.50 and one H-bond acceptor with CYS 331 at distance 2.26 and one H-bond acceptor with ILE322 at distance 3.11 and one H-bond acceptor with THR 333 at distance 3.11 and one H-bond acceptor with HOH 735 at distance 2.52 and one H-bond acceptor with HOH 743 at distance 2.89 shown as hatched line similar to co-crystallized ligand it is give score -14.64.

was done through a force-field MMFF94x Optimization using gradient of 0.0001 for determining low energy conformations with the most favorable (lowest energy) geometry. The crystal structures of inosine-5'-monophosphate dehydrogenase receptor in complex with nicotinamide-adenine-dinucleotide (NAD) were obtained from the Protein Data Bank (PDB) http://www.rcsb.org/pdb/explore. dofistructureId=1NFB (PDB code: 1NFB). Hydrogen atoms and partial charges were added to the protein with the protonation 3D application in MOE. This application is performed to assign ionization states and position of the hydrogen atoms in the macromolecular structure. Most of protein structures obtained from the Protein Data Bank contain little or no hydrogen coordinate data due to limited resolution. Yet, the hydrogen bond network and ionization states can have a dramatic effect on simulations results.

Molecular modeling and analysis of the docked results

The binding free energy was used to rank the binding affinity of the tested compounds to inosine monophosphate dehydrogenase receptor protein. Also, hydrogen bonds between the ligand and amino acids in PTK were used in the ranking of the compounds. Evaluation of the hydrogen bonds was done by measuring the hydrogen bond length which does not exceed 3Å. RMSD of the docking pose compared to the co-crystal ligand position was used in the ranking. The mode of interaction of the native ligand (NAD) within the crystal structure of inosine monophosphate dehydrogenase receptor was used as a standard docked model as well as for RMSD calculation.

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