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Synthesis and structure elucidation of novel fused 1,2,4-triazine derivatives as potent inhibitors targeting CYP1A1 activity ☆

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ABSTRACT

Synthesis and structure elucidation of new series of novel fused 1,2,4-triazine derivatives **3a**–**3f**, **4a**–**4i** and **6a**–**6b** and their inhibitory activities are presented. Molecular structures of the synthesized compounds were confirmed by ^{1}H NMR, ^{13}C NMR, MS spectra and elemental analyses. X-ray crystallographic analysis was performed on 2-acetyl-8-(*N*,*N*-diacetylamino)-6-(4-methoxybenzyl)-3-(4-methoxy-phenyl)-7-oxo-2,3-dihydro-7*H*-[1,2,4]triazolo[4,3-*b*][1,2,4]triazine **3d** and 2-acetyl-8-(*N*-acetyl- amino)-6-benzyl-3-(4-chlorophenyl)-3-methyl-7-oxo-2,3-dihydro-7*H*-[1,2,4]triazolo[4,3-*b*][1,2,4]triazine **4e** to secure their structures. The inhibitory effect of these compounds toward the CPY1A1 activity was screened to determine their potential as promising anticancer drugs. Our data showed that compounds **4e**, **5a**, **5b** and **6b** possess the highest inhibitory effects among all tested compounds. Furthermore, analysis of triazolotriazine derivatives docking showed that these compounds bind only at the interface of substrate recognition site 2 (SRS2) and (SRS6) at the outer surface of the protein. Amino-acids ASN214, SER216 and ILE462 participate in the binding of these compounds through H-bonds.

1. Introduction

In recent years, the screening of natural products and chemical compounds has led to the development of the anticancer drugs currently utilized clinically.[2–8] However, cancer still carries a high mortality rate globally regardless of therapeutic advances. Among the treatment choices available, chemotherapy is one of the most widely used treatment options in spite of its numerous side effects, such as suppression of bone marrow and immune system as well as its development of drug resistance.[9,10] Therefore, the search for new chemo-preventive anticancer agents that are able to not only target chemical carcinogens, but also display increased efficacy and overall decreased systemic toxicity continues to be a great challenge for medical science.

Cytochrome P450s (CYPs) are heme containing isoenzymes that participate in the metabolism of various xenobiotics and endogenous substances. These isoenzymes catalyze a variety of metabolic pathways and thus play an important role primarily in phase I metabolism of xenobiotics facilitating their excretion, as well as in the activation of endogenous compounds to reactive conversion products involved in the regulation of physiological and cellular processes.[11]

CYPs family 1 in particular, are involved in the metabolic activation of numerous chemical carcinogens such as polycyclic aromatic hydrocarbons (PAHs) which are ubiquitous pollutants in human environment. This is the case for benzo[a]pyrene (BP), an infamous environmental PAH considered as a pro-carcinogen for various organs, including breast, lung and skin.[2] The metabolic activation of BP via drug-metabolizing enzymes, such as cytochromes CYP1A1 (aryl hydrocarbon hydroxylase), can produce genotoxic electrophile intermediates that can covalently bind DNA and form mutagenic DNA adducts that might be involved in the initial events of carcinogenesis.[3]

CYPs inhibitors are being considered by many pharmaceutical companies as possible therapeutic targets. Over-expressed CYPs, with altered substrate/inhibitor specificity, offer great advantage for the preparation/formulation of safe anticancer molecules, as

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levels of these CYPs are either undetectable or miniscule in normal cells. Inhibition of these endogenous CYPs in cancerous cells thus might offer a novel target for designing new anticancer agents. Accordingly, various natural and synthetic compounds that are capable of reducing or inhibiting CYPs-mediated bioactivation of PAHs have been reported as potential chemopreventive anticancer agents.[5,6] Among wide variety of natural compounds, flavonoids have traditionally been described as CYP1 inhibitors due to their inhibition of carcinogenic product formation as well as their consequent blockage of the initiation stage of carcinogenesis. One of their most significant characteristics, responsible for their cancer preventive properties, is their selective interaction with cytochrome CYP1 enzymes.[12,13] Moreover, several synthetic compounds have been reviewed for their potency and selectivity as chemical inhibitors for the major human hepatic CYP isoforms. For example, furafylline was found to be the most selective inhibitor available for CYP1A2; 2-phenyl-2-(1-piperidinyl) propane (PPP) for CYP2B6; montelukast for CYP2C8; sulfaphenazole for CYP2C9; (−)-*N*-3-benzyl-phenobarbital for CYP2C19 and quinidine for CYP2D6.[14] Recently, potent CYP17 inhibitors which provide effective treatment of prostate cancer patients were reported.[15] However, more-selective inhibitors are clearly still needed for phenotyping of some CYP isoforms.[14]

Among a wide variety of synthetic compounds being screened for anticancer activities various condensed 1,2,4-triazines have been reported by independent groups to be extremely potent. Sztanke et al. reported that several heterobicyclic systems containing the 1,2,4-triazine moiety have shown significant biological activities. For example the imidazo[2,1-*c*][1,2,4]triazin-4(6*H*)-one core system showed distinctly lower cytotoxicity towards normal cells and several-times higher against cancer cell lines.[16–20] Abdel-Rahman et al. demonstrated that several heterobicyclic nitrogen systems bearing the 1,2,4-triazine moiety possess Leukemia, Lung, Breast and CNS anticancer activities.[21–23] Ciciani et al. synthesized a series of pyrazolo[5,1-*c*][1,2,4]benzotriazine derivatives showing selective cytotoxicity on human colorectal adenocarcinoma cell line HCT-8 in hypoxic as well as normoxic conditions.[24,25] Nevertheless, the role of 1,2,4-triazines derivatives as selective synthetic inhibitors of the major hepatic CYP isoenzymes is yet to be studied.

In our effort to identify new anticancer agents, we synthesized various heterocyclic derivatives bearing the 1,2,4-triazine moiety. Herein, we report syntheses and characterization of the novel compounds as well as their inhibitory activities on CYP1A1.

2. Chemistry

The multi nucleophilic centered molecules, namely, 4-amino-6-substituted benzyl-3-hydrazinyl-1,2,4-triazin-5(4*H*)-one **1a–b** were selected as building block units for the preparation of the target compounds (Fig. 1). A simple synthetic strategy was used to prepare the fused triazines required for the present study. Thus, the reaction of **1** with a variety of one carbon donors, such as substituted benzaldehydes and acetophenones afforded the corresponding Schiff's bases **2a**–**2t** in good yields (Scheme 1).

Cyclocondensation of **2a**–**2t** followed pathway A. Treatment of compound **2a–2t** with acetic anhydride to give either corresponding triacetylated heterobicyclic derivatives 2-acetyl-8-(*N*,*N*-(diacetylamino)-6-substituted benzyl-7-oxo-3-substituted phenyl-2,3-dihydro-7*H*-[1,2,4]-triazolo[4,3-*b*][1,2,4] triazine **3** or diacetylated derivatives **4** (Scheme 2). The structures of these compounds were established by IR, ^{1}H NMR, ^{13}C NMR, HMQC, and elemental analysis. Even though their structures can be inferred from their spectroscopic and analytical data, the structures of **3d** and **4e** were secured by X-ray diffraction analysis as prototypes (Tables 1 and 2; Figs. 2 and 3). The structure of **4e** is centrosymmetric, and hence racemic. There are two crystallographically independent molecules A (R on C3) and B (S on C3) in the asymmetric unit (Fig. 3).

Another attempt for cyclocondensation was performed by allowing 4-amino-6-substituted benzyl-3-hydrazinyl-1,2,4-triazin-5(4*H*)-one **1** to react with isatin as an example for 1,2-dicarbonyl analogue. In this case, condensation occurred only between the isatin ketonic carbonyl group and the NH_2 group of the hydrazine moiety of **1** to form the hydrazone **5** (Scheme 3). The structures of compounds **5a** and **5b** were confirmed by their IR, ^{1}H

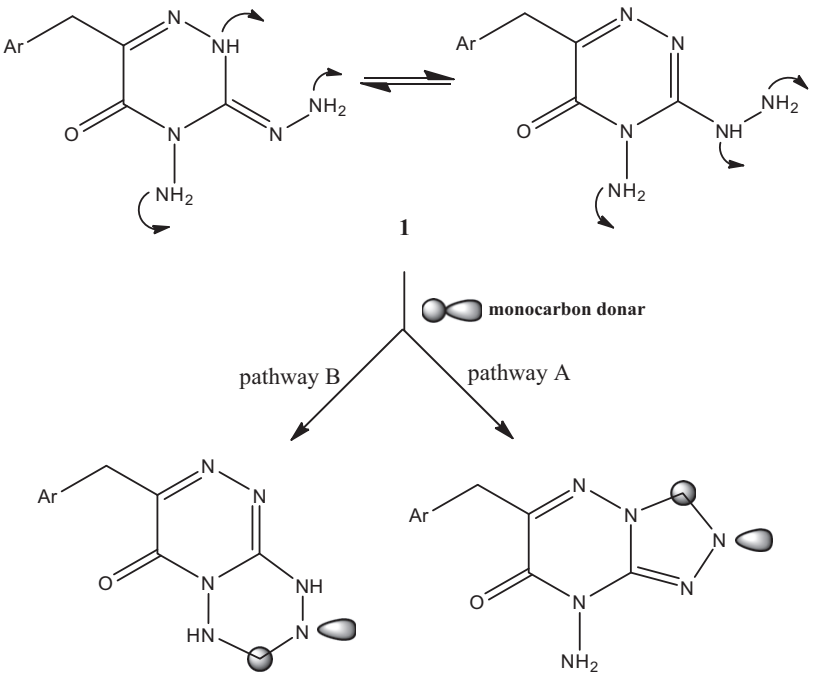
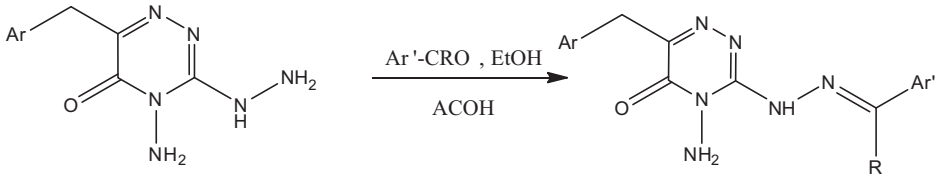


Figure 1. Pathways for the annelation reaction of **1** with monocarbon donor.



1

	Ar
1a	C_6H_5
1b	4-OCH_3-C_6H_4

2

	R	Ar	Ar'
2a	H	C_6H_5	C_6H_5
2b	H	4-OCH_3-C_6H_4	C_6H_5
2c	H	C_6H_5	4-OCH_3-C_6H_4
2d	H	4-OCH_3-C_6H_4	4-OCH_3-C_6H_4
2e	H	C_6H_5	3,4,5-$(OCH_3)_3$-C_6H_2
2f	H	4-OCH_3-C_6H_4	3,4,5-$(OCH_3)_3$-C_6H_2
2g	H	C_6H_5	4-OH-C_6H_4
2h	H	4-OCH_3-C_6H_4	4-OH-C_6H_4
2i	H	C_6H_5	4-NO_2-C_6H
2j	H	4-OCH_3-C_6H_4	4-NO_2-C_6H
2k	CH_3	C_6H_5	C_6H_5
2l	CH_3	4-OCH_3-C_6H_4	C_6H_5
2m	CH_3	C_6H_5	4-Cl-C_6H_4
2n	CH_3	4-OCH_3-C_6H_4	4-Cl-C_6H_4
2o	CH_3	C6H5	4-CH_3-C_6H_4
2p	CH_3	4-OCH_3-C_6H_4	4-CH_3-C_6H_4
2q	CH_3	C_6H_5	2-pyridyl
2r	CH_3	4-OCH_3-C_6H_4	2-pyridyl
2s	CH_3	C_6H_5	2-furyl
2t	CH_3	4-OCH_3-C_6H_4	2-furyl

Scheme 1. General synthesis of compound **2**.

NMR, ^{13}C NMR and elemental analysis. In turn, these compounds have undergone acylation with acetic anhydride to afford the corresponding spiro compound **6a** and **6b** as evident from their IR, ^{1}H NMR, ^{13}C NMR and elemental analysis (Scheme 3). In ^{1}H NMR spectrum of **6a** four methyl groups beside the benzylic and aromatic protons were observed (see Section 5). Furthermore, the structure was supported by ^{13}C NMR which showed the spiro quaternary carbon at δ 83.06. Similarly, the structure of **6b** was confirmed. Based on the number of NMR signals for methyl groups, the structural possibilities like **7** and **8** were ruled out.

The condensation reaction between **1** and triethyl orthoformate as the monocarbon donor in methanol and the presence of catalytic amount of acetic acid furnished [1,2,4]triazolo[4,3-*b*][1,2,4]triazin-7(8*H*)-one **9** (Scheme 4). The assigned structures of the reaction products such as **9** rather than **10** were based on their spectroscopical data. Thus, the IR spectra of **9a** and **9b** were quite distinct allowing the observation of the characteristic peaks for the NH_2 groups at 3321, 3220 and 3292, 3241 cm^{-1}, respectively, and supported by ^{1}H NMR that revealed the exchangeable protons at δ 5.94 and 6.03 with two protons intensity each.

However, the reaction of **1** with acetic anhydride led to the formation of the 1*H*-[1,2,4]triazino[4,3-*b*][1,2,4,5]tetrazin-6(4*H*)-one **11** via pathway B. The structures of **11a** and **11b** were confirmed using NMR experiments including NOE together with their elemental analyses. For example, in the NOE spectrum of **11a** a correlation was observed between the two methyl groups of 91.86% indicating the short distance between them and thus eliminating structure **12** for the reaction product.

In order to test the capability of the multiacetylated compounds **3** and **6** as acetyl donors, compounds **3c**, **3d** and **6a** as prototype were allowed to react with *o*-phenylenediamine (*o*-PDA) in ethanol and the reaction was monitored by TLC which detected the formation of 2-methyl-1*H*-benzo[*d*]imidazole. In case of compound **3c** the reaction mixture of was worked up to isolate the diacetylated analogue **4j** and 2-methyl-1*H*-benzo[*d*]imidazole.

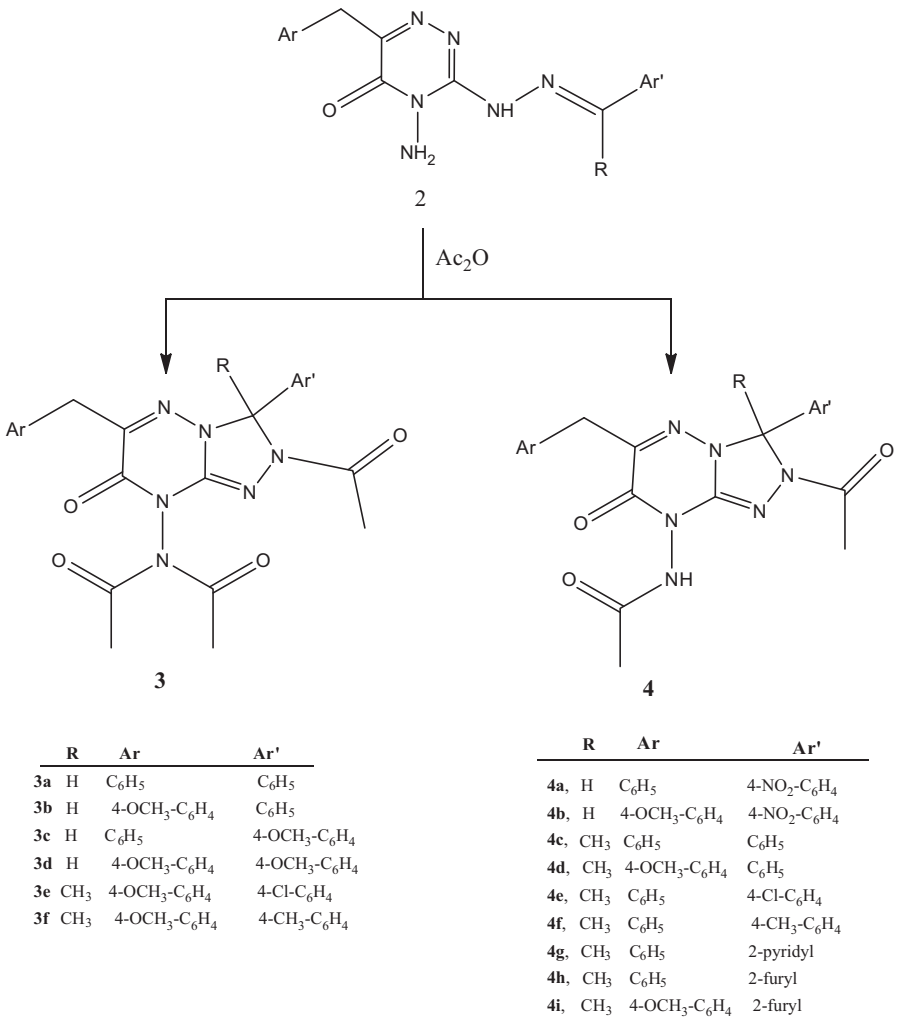
3. Biology

3.1. Microsomal aryl hydrocarbon hydroxylase activity

Microsomal aryl hydrocarbon hydroxylase (AHH) activity was determined with or without triazolotriazine derivatives and percentage of changes in AHH activities were calculated according to the methods reported earlier.[26–28] Data presented in Table 3 showed that triazolotriazine derivatives affect the aryl hydrocarbon hydroxylase (AHH) activity in different manner; marked inhibitory effects were seen with **5a** (92.23% ± 2.07), **5b** (90.45% ± 2.25), **4e** (87.90% ± 2.30) and **6b** (81.57% ± 1.9) whereas moderated inhibitory effects were detected with **4f** (75.58% ± 0.64), **6a** (64.56% ± 4.54), **4d** (59.81% ± 4.26), **3b** (57.59% ± 2.65) and **3c** (51.47% ± 0.64). Nevertheless, low inhibition effect were detected with **3a** (20.63 ± 3.32), **4c** (37.92 ± 3.41) and **3f** (48.16 ± 3.55). Table 3 also showed that compound **5a** exhibits the lowest IC_{50} value. In the present study, we report for the first time to the best of our knowledge that 1,2,4-triazine derivatives, constitute potent inhibitors of drug-metabolizing CYP 450 enzymes. These data showed that compounds with **5a**, **5b**, **4e** and **6b** may be identified as a constituting new chemical class of CYP1A1 inhibitors.

3.2. Acute toxicity test

Oral and intraperitoneal acute toxicity test results indicated that compounds **5a**, **5b 4e** and **6b** proved to be non-toxic and well tolerated by the experimental animals up to 350 and 150 mg/kg in oral and intraperitoneal acute toxicity test, respectively. Based on these results, the single acute oral LD_{50} of the tested compounds is greater than 350 mg/kg of body weight.



	R	Ar	Ar'
3a	H	C_6H_5	C_6H_5
3b	H	4-OCH_3-C_6H_4	C_6H_5
3c	H	C_6H_5	4-OCH_3-C_6H_4
3d	H	4-OCH_3-C_6H_4	4-OCH_3-C_6H_4
3e	CH_3	4-OCH_3-C_6H_4	4-Cl-C_6H_4
3f	CH_3	4-OCH_3-C_6H_4	4-CH_3-C_6H_4

	R	Ar	Ar'
4a,	H	C_6H_5	4-NO_2-C_6H_4
4b,	H	4-OCH_3-C_6H_4	4-NO_2-C_6H_4
4c,	CH_3	C_6H_5	C_6H_5
4d,	CH_3	4-OCH_3-C_6H_4	C_6H_5
4e,	CH_3	C_6H_5	4-Cl-C_6H_4
4f,	CH_3	C_6H_5	4-CH_3-C_6H_4
4g,	CH_3	C_6H_5	2-pyridyl
4h,	CH_3	C_6H_5	2-furyl
4i,	CH_3	4-OCH_3-C_6H_4	2-furyl

Scheme 2. General synthesis of compounds **3** and **4**.

4. Structural modeling and inhibitors docking

CYP1A1 3D structure model was generated using sequence-sequence comparative modeling. Through PSI-BLAST, CYP1A2 (PDB: 2hi4) was determined as the most suitable template for predicting CYP1A1 structure based on a sequence identity of 73.5%. The validity of the obtained structure was confirmed by Raman plot which showed only 0.5% of the amino acids in the disallowed region (data not shown). The predicted CYP1A1 structure was then used through EAdock[29] and SwissDock[30] to predict binding modes of alpha-naphthoflavone (ANF) and the triazolotriazine derivatives (i.e., **4e**, **5a**, **5b**, **6b**). Binding modes were analyzed using UCSF chimera.[31] According to the obtained data (Fig. 4A–E) none of triazolotriazine derivatives gave a binding mode in the active site. This is not surprising. Based on the structure of CYP1A2 active site[32] and the sequence identity between CYP1A2 and CYP1A1, the active site structure of CYP1A1 will be highly adapted for positioning and oxidation of relatively large, planar substrates. Furthermore, CYP1A1 substrates will be bulkier than those of CYP1A2.[32] However; this is not the case of our studied compounds. The lowest fullfitness energy indicates that the most probable binding mode for all the studied triazolotriazine derivatives is at the interface between SRS2 (Fig. 4, purple) and SRS6 (Fig. 4, blue) at the outer surface of the protein. This finding raised the question about the possibility that this docking site (i.e., SRS2/SRS6) is accessible in nature by substrates and/or inhibitors. To answer this question, binding mode simulation was carried out with ANF which is a natural substrate for CYP1A1, a natural inhibitor of CYP1A2[32–34] and has already been used in studying CYP1A2 3D structure (PDB: 2hi4).[32] It was found that, besides binding the active site, ANF binds the SRS2/SRS6 site as for triazolotriazine derivatives (Fig. 4F). Furthermore, the docking fullfitness energy difference between the active site and the SRS2/SRS6 site was not significant (Table 4) since it was less than 5 kcal/mol. This indicates that ANF binds freely to the active site, the SRS2/SRS6 site or both of them. If this information is true, it can be assumed that at the molecular level more than one triazolotriazine derivative will bind to the enzyme. Consequently the obtained data confirm the assumption of the presence of two or more substrate binding sites and the allosterism of CYP1A1.[35] This information also indicates that certain sites outside the active site play a role in the activity of CYP1A1. One of these probable sites is SRS2/SRS6 site. The probable role may be through the modification of the enzyme 3D structure due to binding to the SRS2/SRS6 site, leading to reduction or prevention of binding of the substrate to the active site. This can, in part, explains the mechanism by which triazolotriazine derivatives inhibit CYP1A1.

However, the difference in the inhibitory potential of the studied triazolotriazine derivatives remains to be explained. Therefore, further analysis was carried out to determine the regions that par-

Table 1
Crystal data and structure refinement for **3d**

Empirical formula	$C_{25}H_{26}N_6O_6$
Formula weight	506.52
Temperature	173(2) K
Wavelength	0.71073 Å
Crystal system	Triclinic
Space group	$P\bar{1}$
Unit cell dimensions	a = 9.4281(5) Å; b = 10.2953(5) Å; c = 12.6745(7) Å
Angles α, β, γ	91.896(1)°, 103.660(1)°, 90.882(1)°
Volume	1194.46(11) Å^3
Z	2
Density (calculated)	1.408 Mg/m^3
Absorption coefficient	0.103 mm^{-1}
F(000)	532
Crystal size	0.32 × 0.30 × 0.20 mm^3
Theta range for data collection	2.44 to 25.10°
Index ranges	−11<=h<=11, −12<=k<=12, −15<=l<=15
Reflections collected	13170
Independent reflections	4247 [R(int) = 0.0735]
Completeness to theta = 25.10°	99.9%
Absorption correction	Multi-scan
Max. and min. transmission	0.9797 and 0.9677
Refinement method	Full-matrix least-squares on F^2
Data/restraints/parameters	4247/0/339
Goodness-of-fit on F^2	0.999
Final R indices [I >2σ(I)]	R1 = 0.0548, wR2 = 0.1427
R indices (all data)	R1 = 0.0790, wR2 = 0.1585
Largest diff. peak and hole	0.234 and −0.313 e.Å^{-3}

Table 2
Crystal data and structure refinement for **4e**

Empirical formula	$C_{22}H_{21}ClN_6O_3$
Formula weight	452.90
Temperature	153(2) K
Wavelength	0.71073 Å
Crystal system	Monoclinic
Space group	$P2_1/n$
Unit cell dimensions	a = 10.3866(5) Å; b = 15.8554(8) Å; c = 26.7122(14) Å
Angles α, β, γ	90°, 93.763(1)°, 90°
Volume	4389.6(4) Å^3
Z	8
Density (calculated)	1.371 Mg/m^3
Absorption coefficient	0.211 mm^{-1}
F(000)	1888
Crystal size	0.42 × 0.24 × 0.11 mm^3
Theta range for data collection	2.00 to 25.39°
Index ranges	−12<=h<=12, −19<=k<=19, −32<=l<=32
Reflections collected	48135
Independent reflections	8063 [R(int) = 0.1024]
Completeness to theta = 25.39°	99.9%
Absorption correction	Multi-scan
Max. and min. transmission	0.9771 and 0.9165
Refinement method	Full-matrix least-squares on F^2
Data/restraints/parameters	8063/0/583
Goodness-of-fit on F^2	1.022
Final R indices [I >2sigma(I)]	R1 = 0.0637, wR2 = 0.1584
R indices (all data)	R1 = 0.1104, wR2 = 0.1836
Largest diff. peak and hole	0.533 and −0.512 e.Å^{-3}

ticipate in the interaction between the SRS2/SRS6 site and triazolotriazine derivatives (Fig. 5). These regions were found to be ILE18-LEU30, ASN191-PHE220 and ILE462-LEU465. According to the binding mode of the triazolotriazine derivatives, the amino acids in the vicinity of the SRS/SRS6 site that can interact with the triazolotriazine derivatives will change. It was found that certain amino acids are involved in the triazolotriazine derivatives binding modes by H-bond formation. These amino acids are ASN214, SER216 and ILE462. The possible binding modes are determined by the fullfitness energy range in Table 4. According to these findings, one can assume that the position of amino acids and the extent of their implication in fixing the triazolotriazine derivatives will determine the degree of change in enzyme structure and hence its function. The obtained data can partially explain the difference in the inhibition potential of the triazolotriazine derivatives (Table 4). Further studies are now being carried out to determine the role of the assumed amino acids in CYP1A1 activity.

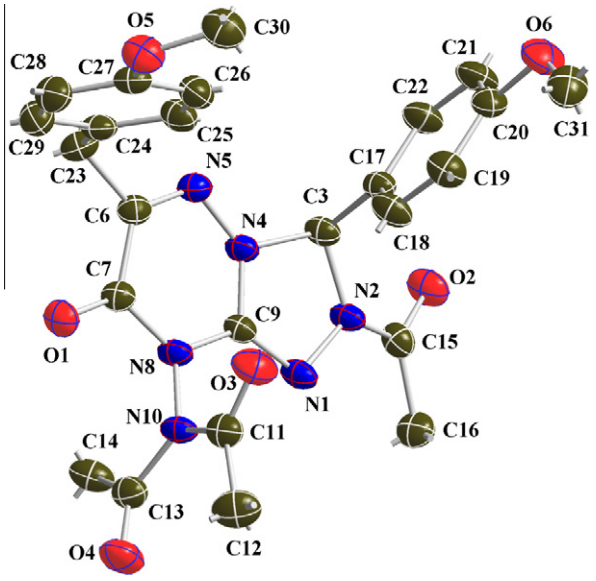


Figure 2. Numbering scheme with atomic displacement ellipsoids drawn at 50% probability level of compound **3d**.

In summary, our results suggest that compounds such **4e**, **5a**, **5b** and **6b** may be considered as leads for chemoprevention anticancer studies.

5. Experimental protocols

5.1. Chemistry

Melting points were determined with a Mel-Temp apparatus and are uncorrected. Magnetic resonance spectra (^{1}H NMR and ^{13}C NMR spectra) were recorded using a JEOL 500 MHz spectrometer with the chemical shift values reported in δ units (part per million). Infrared data were obtained using a Perkin–Elmer 1600 series Fourier transform instrument as KBr pellets. The compounds were named using Chem. Draw Ultra version 12, Cambridge soft Corporation. Follow up of the reactions and checking the purity of the compounds were made by TLC on silica gel-protected aluminum sheets (Type 60 GF254, Merck) and the spots were detected by exposure to UV-lamp at λ 254 nm for few seconds. X-ray diffraction data were collected using a Siemens SMART CCD diffractometer with Mo–Kα radiation (λ = 0.71073 Å, graphite monochromator). The crystals were cooled to 173(2)K for **3d** and 153(2)K for **4e** by a flow of nitrogen gas using the LT-2A device. A full sphere of reciprocal space was scanned by 0.3 steps in ω with a crystal-to-detector distance of 3.97 cm. Elemental analyses were performed on Perkin–Elmer 2400 elemental analyzer, and the obtained values were within ±0.3% of the theoretical values.

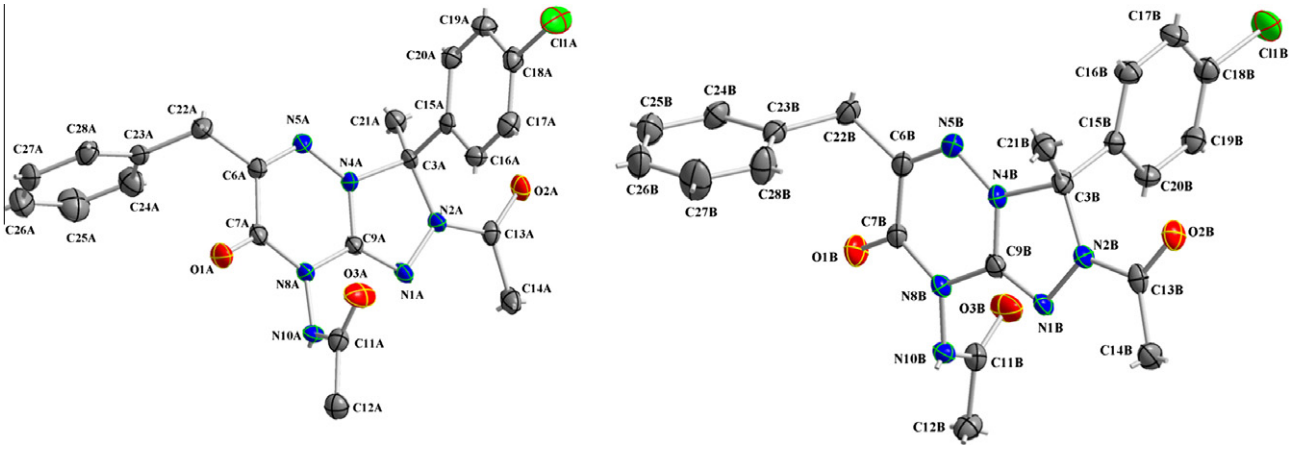
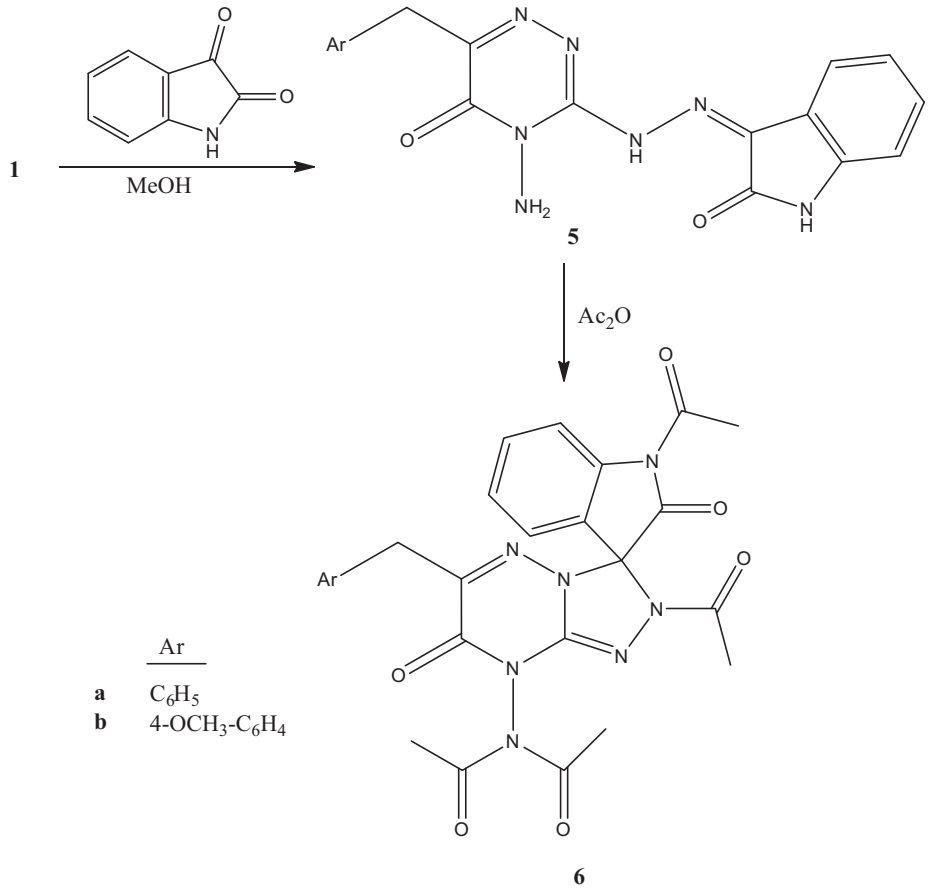


Figure 3. A perspective drawing of molecules A and B of compound **4e**, showing the atom-numbering scheme. Displacement ellipsoids are drawn at 50% probability level.



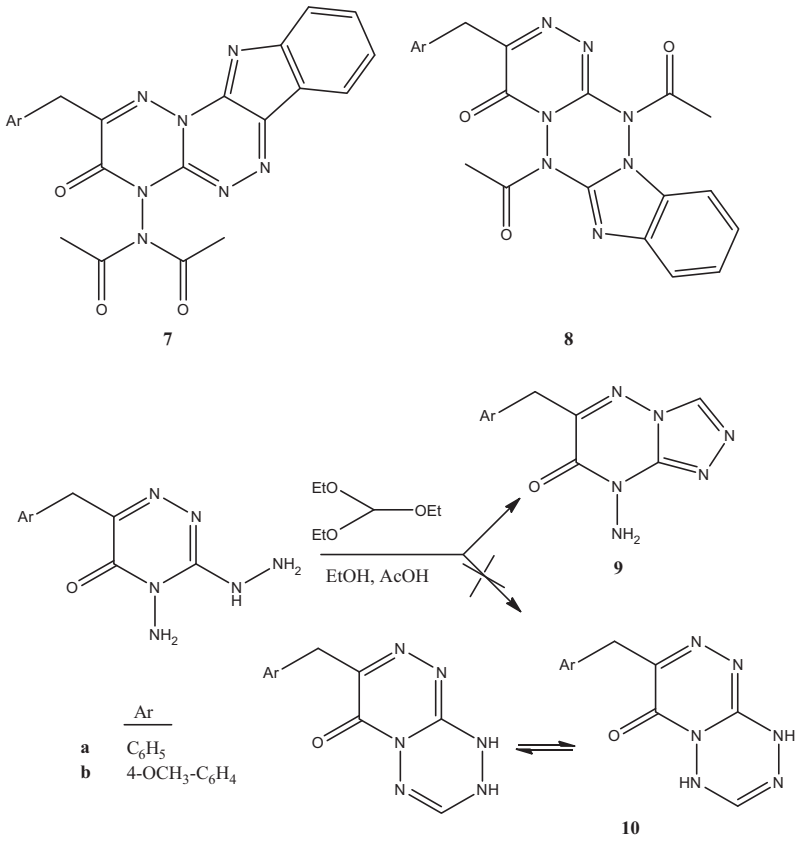
Scheme 3. Synthesis of compounds **5** and **6**.

5.2. General procedure for preparation of 4-amino-6-substituted benzyl-3-hydrazinyl-1,2,4-triazin-5(4*H*)-one (1)

To a solution of 4-amino-6-aryl-3-(methylthio)-1,2,4-triazin-5(4*H*)-one (1.6 mmol) in 5 mL isopropyl alcohol, 1 mL hydrazine hydrate (80%) was added. The reaction mixture was refluxed for about 3 h then cooled to room temperature. The product that precipitated out was filtered off and washed well with ethanol.[36]

5.2.1. 4-Amino-6-benzyl-3-hydrazinyl-1,2,4-triazin-5(4*H*)-one (1a)

Compound **1a** was obtained as pale yellow needles, 0.21 g (53.8%) yield, mp—(°C) 194–195 (Lit. mp (°C)—254–255).[36] ^{1}H NMR (500 MHz: $CDCl_3$): δ: 3.89 (s, 2H, CH_2), 4.38 (s. 2H, NH_2, D_2O exchangeable), 5.51 (s, 2H, NH_2, D_2O exchangeable), 7.13-7.16 (m, 1H, Ar-H), 7.22 (d, 4H, J = 3.8 Hz, Ar-H), 8.18 (s, 1H, NH, D_2O exchangeable).



Scheme 4. Synthesis of compound **9**.

Table 3
The effect of triazolotriazine derivatives on the aryl hydrocarbon hydroxylase activity (AHH)

Code	Ar	Ar′	% Inhibition	IC_{50} M
2d	4-OCH_3-C_6H_4	4-OCH_3-C_6H_4	nd	nd
3a	C_6H_5	C_6H_5	20.63 ± 3.32	2.42×10^{-8}
3b	4-OCH_3-C_6H_4	C_6H_5	57.59 ± 2.65	8.67×10^{-8}
3c	C_6H_5-	4-OCH_3-C_6H_4	51.47 ± 0.64	9.71×10^{-9}
3f	4-OCH_3-C_6H_4	4-CH_3-C_6H_4	48.16 ± 3.55	1.04×10^{-8}
4c	C_6H_5	C_6H_5	37.92 ± 3.41	1.32×10^{-8}
4d	4-OCH_3-C_6H_4	C_6H_5	59.81 ± 4.26	8.36×10^{-9}
4e	C_6H_5	4-Cl-C_6H_4	87.90 ± 2.30	5.69×10^{-9}
4f	C_6H_5	4-CH_3-C_6H_4	75.58 ± 0.64	6.62×10^{-9}
5a	C_6H_5		92.23 ± 2.07	5.42×10^{-9}
5b	4-OCH_3-C_6H_4		90.45 ± 2.25	5.53×10^{-9}
6a	C_6H_5		64.56 ± 4.54	7.74×10^{-9}
6b	4-OCH_3-C_6H_4		81.54 ± 1.9	6.14×10^{-8}

The results were expressed as mean ± S.E.M. Data were analyzed by one way of variance. Student's *t* test for unpaired observations was used. *p* value <0.001 and was significant. Number of experiments was 3.

5.2.2. 4-Amino-6-(4-methoxybenzyl)-3-hydrazinyl-1,2,4-triazin-5(4*H*)-one (1b)

Compound **1b** was obtained as yellow needles, 0.35 g (87.5%) yield, mp (°C)—258–259 °C (Lit. mp (°C)—243–244).[36]

5.3. General procedure for the reaction of 4-amino-6-aryl-3-hydrazinyl-1,2,4-triazin-5(4*H*)-one (1) with aldehydes and acetophenones

To a solution of 4-amino-6-benzyl-3-hydrazinyl-1,2,4-triazin-5(4*H*)-one **1a** (2.5 mmol) or 4-amino-6-(4-methoxybenzyl)-3-hydrazinyl-1,2,4-triazin-5(4*H*)-one **1b** (2.5 mmol) in 10 mL ethanol, the corresponding aldehyde (2.5 mmol) or acetophenone (2.5 mmol) was added and heated under reflux for 2 h. The product obtained on cooling was filtered off and recrystallized from ethanol.

5.3.1. 4-Amino-6-benzyl-3-(2-benzylidenehydrazinyl)-1,2,4-triazin-5(4*H*)-one (2a)

Compound **2a** was obtained as yellow crystals, 0.69 g (95.8%) yield, mp (°C)—198–199. IR (KBr): 3302, 3232 (NH_2+NH), 1650(NCO) cm^{-1}. ^1H NMR (500 MHz: DMSO-d_6): δ 3.84 (s, 2H, CH_2), 5.76 (s, 2H, NH_2, D_2O exchangeable), 7.17–7.36 (m, 8H, Ar-H), 7.90 (d, 2H, J = 6.1 Hz, Ar-H), 8.27 (s, 1H, CH), 12.26 (s, 1H, NH, D_2O exchangeable). Anal. Calcd for $C_{17}H_{16}N_6O$: C, 63.74; H, 5.03; N, 26.23. Found: C, 63.55; H, 5.27; N, 26.01.

5.3.2. 4-Amino-3-(2-benzylidenehydrazinyl)-6-(4-methoxybenzyl)-1,2,4-triazin-5(4*H*)-one (2b)

Compound **2b** was collected as yellow crystals, 0.52 g (81.1%) yield, mp (°C)—166–161. IR (KBr): 3288, 3229 (NH_2+NH), 1666 (NCO) cm^{-1}. ^1H NMR (500 MHz: DMSO-d_6): δ 3.70 (s, 3H, OCH_3), 3.76 (s, 2H, CH_2), 5.75 (s, 2H, NH_2, D_2O exchangeable), 6.82 (d, 2H, J = 8.4 Hz, Ar-H), 7.15 (d, 2H, J = 9.2 Hz, Ar-H), 7.37–7.38 (m, 3H, Ar-H), 7.90 (d, 2H, J = 6.1 Hz, Ar-H), 8.27 (s, 1H, CH), 12.31 (s, 1H, NH, D_2O exchangeable). Anal. Calcd for $C_{18}H_{18}N_6O_2$: C, 61.70; H, 5.18; N, 23.99. Found: C, 61.14; H, 5.41; N, 23.78.

5.3.3. 4-Amino-6-benzyl-3-(2-(4-methoxybenzylidene)-hydrazinyl)-1,2,4-triazin-5(4*H*)-one (2c)

Compound **2c** was obtained as yellow crystals, 0.69 g (87.9%) yield, mp (°C)—150–151. IR (KBr): 3308, 3219 (NH_2+NH), 1650 (NCO) cm^{-1}. ^1H NMR (500 MHz: DMSO-d_6): δ 3.77 (s, 3H, OCH_3), 3.91 (s, 2H, CH_2), 6.96 (d, 2H, J = 8.4 Hz, Ar-H), 7.14–7.22 (m, 2H,

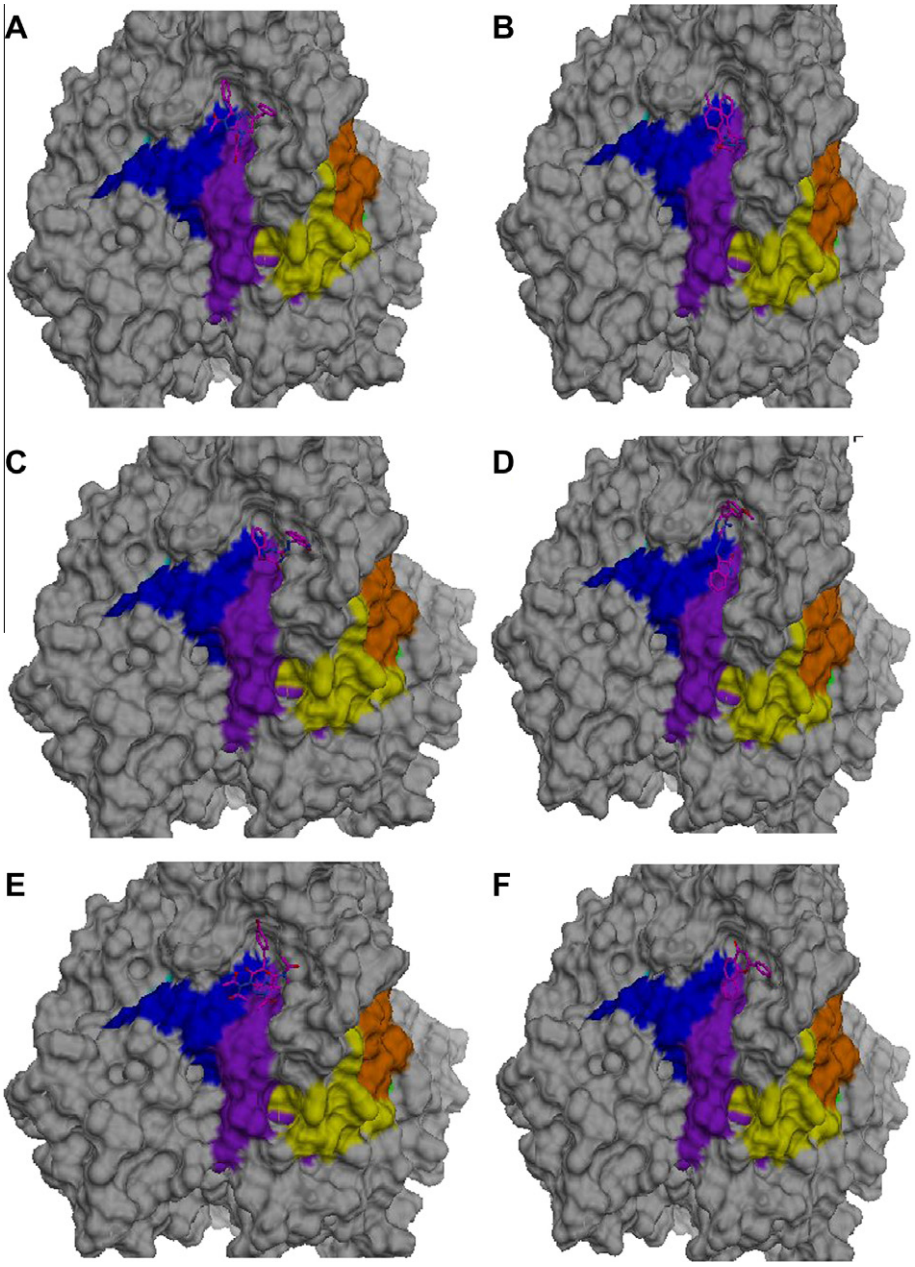


Figure 4. Binding mode of triazolotriazine derivatives to CYP1A1 in comparison to ANF. CYP1A1 is shown in gray. The hetero-compounds are the triazolotriazine derivatives. **3a** derivative, (A) **4e** derivative, (B) **5a** derivative, (C) **5b** derivative, (D) **6b** derivative, (E) and ANF, (F) Substrate recognition sites (SRS) are colored as follows: SRS1, orange; SRS2, purple; SRS3, yellow; SRS4, green; SRS5, cyan; SRS6, blue.

NH_2, D_2O exchangeable), 7.28–7.30 (m, 5H, Ar-H), 7.87 (d, 2H, J = 8.4 Hz, Ar-H), 8.41 (s, 1H, CH), 12.20 (s, 1H, NH, D_2O exchangeable). Anal. Calcd for $C_{18}H_{18}N_6O_2$: C, 61.70; H, 5.18; N, 23.99. Found: C, 61.42.; H, 5.46; N, 23.74.

5.3.4. 4-Amino-6-(4-methoxybenzyl)-3-(2-(4-methoxybenzyl idene)hydrazinyl)-1,2,4-triazin-5(4*H*)-one (2d)

Compound **2d** was obtained as yellow crystals, 0.77 g (89.6%) yield, mp (°C)—150–151. IR (KBr): 3331, 3244 (NH_2+NH), 1650 (NCO) cm^{-1}. ^1H NMR (500 MHz: DMSO-d_6): δ 3.67 (s, 2H, CH_2), 3.76 (s, 6H, OCH_3), 5.72 (s, 2H, NH_2, D_2O exchangeable), 6.81 (d, 2H, J = 8.4 Hz, Ar-H), 6.92 (d, 2H, J = 8.4 Hz, Ar-H.), 7.15(d, 2H, J = 8.4 Hz, Ar-H), 7.84 (d, 2H, J = 8.4 Hz, Ar-H), 8.21 (s, 1H, CH), 12.24 (s, 1H, NH, D_2O exchangeable). Anal. Calcd for $C_{19}H_{20}N_6O_3$: C, 59.99; H, 5.30; N, 22.09. Found: C, 75.2; H, 5.58; N, 22.124.

5.3.5. 4-Amino-6-benzyl-3-(2-(3,4,5-trimethoxybenzylidene)-hydrazinyl)-1,2,4-triazin-5(4*H*)-one (2e)

Compound **2e** was obtained as pale yellow crystals, 0.93 g (98.9%) yield, mp (°C)—157–158. IR (KBr): 3310, 3224 (NH_2+NH), 1682 (NCO) cm^{-1}. ^1H NMR (500 MHz: DMSO): δ 3.35 (s, 3H, OCH_3), 3.80 (s, 6H, OCH_3), 3.85 (s, 2H, CH_2), 5.76 (s, 2H, NH_2, D_2O exchangeable), 7.28–7.25 (m, 7H, Ar-H), 8.18 (s, 1H, CH), 12.35 (s, 1H, NH, D_2O exchangeable). Anal. Calcd for $C_{20}H_{22}N_6O_4$: C, 58.53; H, 5.40; N, 20.48. Found: C, 58.97; H, 5.14; N, 20.32.

5.3.6. 4-Amino-6-(4-methoxybenzyl)-3-(2-(3,4,5-trimethoxy-benzylidene)hydrazinyl)-1,2,4-triazin-5(4*H*)-one (2f)

Compound **2f** was obtained as pale yellow crystals, 0.81 g (92%) yield, mp (°C)—100–101. IR (KBr): 3308, 3229 (NH_2+NH), 1676 (NCO) cm^{-1}. ^1H NMR (500 MHz: DMSO): δ 3.64–3.65 (m, 12H,

Table 4
Docking fullfitness energy for the triazolotriazine derivatives and alpha-naphthoflavone (ANF) on both the susbtrate recognition sites (SRS) and the active site of CYP1A1. The fullfitness energy range is related to all the possible binding modes that have no significant difference in the energy level and can take place at the SRS2/SRS6 site

Code	Fullfitness energy (kcal/mol)		% Inhibition
	SRS2/SRS6 site[b]	Active site	
3a	−1436 to −1431	NA[c]	20.63 ± 3.32
6b	−1417 to −1412	NA	81.54 ± 1.90
4e	−1420 to −1415	NA	87.90 ± 2.30
5b	−1384 to −1376	NA	90.45 ± 2.25
5a	−1383 to −1378	NA	92.23 ± 2.07
ANF[a]	−1463 to −1458	−1461	NA

[a] Alpha-naphthoflavone.
[b] SRS = Substrate recognition sites. The energy range is due to the possible docking models of the hetero-compound to CYP1A1.
[c] NA = not applicable.

OCH_3), 3.66 (s, 2H, CH_2), 5.75 (s, 2H, NH_2, D_2O exchangeable), 6.77, 6.81 (2d, 2H, J = 8.4H, Ar-H), 7.09, 7.13 (2d, 2H, J = 8.4 Hz, Ar-H), 7.23 (s, 1H, Ar-H), 7.42 (s, 1H, Ar-H), 8.25 (s, 1H, CH), 12.34 (s, 1H, NH, D_2O exchangeable). Anal. Calcd for $C_{21}H_{24}N_6O_5$: C, 57.26; H, 5.49; N, 19.08. Found: C, 57.02; H, 5.62; N, 19.33.

5.3.7. 4-Amino-6-benzyl-3-(2-(4-Hydroxybenzylidene)-hydrazinyl)-1,2,4-triazin-5(4*H*)-one (2g)

Compound **2g** was obtained as pale yellow crystals, 0.73 g (96.1%) yield, mp (°C)—228–229. IR (KBr): 3425, 3292, 3180 (NH_2+NH), 3425–3180 (br, OH), 1683 (NCO) cm^{-1}. ^1H NMR (500 MHz: DMSO-d_6): δ 3.82 (s, 2H, CH_2), 4.04 (s, 1H, OH, D_2O exchangeable), 5.71 (s, 2H, NH_2, D_2O exchangeable), 6.73 (d, 2H, J = 8.4 Hz, Ar-H), 7.18–7.28 (m, 5H, Ar-H), 7.71 (d, 2H, J = 8.4 Hz, Ar-H), 8.15 (s, 1H, CH), 13.14 (s, 1H, NH, D_2O exchangeable). Anal. Calcd for $C_{17}H_{16}N_6O_2$: C, 60.71; H, 4.79; N, 24.99. Found: C, 60.43.; H, 5.04; N, 24.70.

5.3.8. 4-Amino-3-(2-(4-hydroxybenzylidene)hydrazinyl)-6-(4-methoxybenzyl)-1,2,4-triazin-5(4*H*)-one (2h)

Compound **2h** was obtained as pale yellow crystals, 0.42 g (49.7%) yield, mp (°C)—210–211. IR (KBr): 3276, 3136 (NH_2+NH), 3276–2943 (br, OH), 1686 (NCO) cm^{-1}. ^1H NMR (500 MHz: DMSO-d_6): δ 3.67 (s, 3H, OCH_3); 3.75 (s, 2H, CH_2), 4.04 (s, 1H, OH, D_2O exchangeable), 5.71 (s, 2H, NH_2, D_2O exchangeable), 6.73 (d, 2H, J = 8.4 Hz, Ar-H), 6.81 (d, 2H, J = 8.4 Hz, Ar-H), 7.15 (d, 2H, J = 8.4 Hz, Ar-H), 7.73 (d, 2H, J = 8.4 Hz, Ar-H), 8.10 (s, 1H, CH), 12.17(s, 1H, NH, D_2O exchangeable). Anal. Calcd for $C_{18}H_{18}N_6O_3$: C, 59.01; H, 4.95; N, 22.94. Found: C, 59.27; H, 4.76; N, 22.77.

5.3.9. 4-Amino-6-benzyl-3-(2-(4-nitrobenzylidene)hydrazinyl)-1,2,4-triazin-5(4*H*)-one (2i)

Compound **2i** was obtained as yellow crystals, 0.84 g (96.1%) yield, mp (°C)—180–181. UV/VIS (CH_3CN) λ_{max} = 234 nm, ε = 235,500; λ_{max} = 289 nm, ε = 62,300. IR (KBr): 3331, 3223 (NH_2+NH), 1691 (NCO), 1522, 1321 (NO_2) cm^{-1}; ^1H NMR (500 MHz: DMSO-d_6): δ 3.86 (s, 2H, CH_2), 5.81 (s, 2H, NH_2,D_2O exchangeable), 7.18–7.29 (m, 5H, Ar-H), 8.17–8.2 (m, 4H, Ar-H), 8.37 (s, 1H, CH), 12.58 (s, 1H, NH, D_2O exchangeable). Anal. Calcd for $C_{17}H_{15}N_7O_3$: C, 55.89; H, 4.14; N, 26.84. Found: C, 55.60; H, 4.42; N, 26.63.

5.3.10. 4-Amino-6-(4-methoxybenzyl)-3-(2-(4-nitrobenzylidene)hydrazinyl)-1,2,4-triazin-5(4*H*)-one(2j)

Compound **2j** was obtained as orange yellow crystals, 0.8 g (87.2%) yield, mp (°C)—195–196. IR (KBr): 3385, 3306 (NH_2+NH), 1641 (NCO), 1524, 1323 (NO_2) cm^{-1}. ^1H NMR (500 MHz: DMSO-d_6): δ 3.68 (s, 2H, CH_2), 3.73 (s, 3H, OCH_3), 5.81 (s, 2H, NH_2, D_2O exchangeable), 6.82 (d, 2H, J = 8.4 Hz, Ar-H), 7.16 (d, 2H, J = 8.4 Hz, Ar-H), 8.17–8.24 (m, 4H, Ar-H), 8.38 (s, 1H, CH), 12.56 (s, 1H, NH, D_2O exchangeable). Anal. Calcd for $C_{18}H_{17}N_7O_4$: C, 54.68; H, 4.33; N, 24.80. Found: C, 54.42; H, 4.62; N, 24.90.

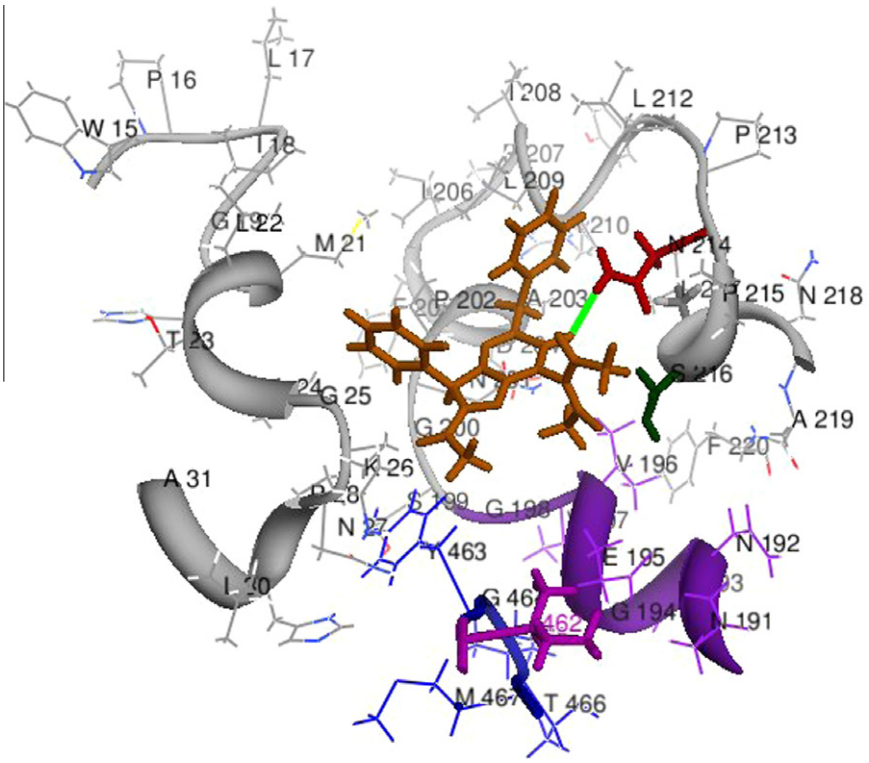


Figure 5. The amino acid structure of the SRS2/SRS6 site. SRS2, purple; SRS6, blue; asn214, red; ser216, dark green; ile462, magenta and other amino acids are shown in gray. H-bond between HD21 of asn214 (red) and oxygen from triazolotriazine derivative (orange) is shown in light green.

5.3.11. 4-Amino-6-benzyl-3-(2-(1-phenylethylidene)-hydrazinyl)-1,2,4-triazin-5(4*H*)-one (2k)

Compound **2k** was obtained as yellow crystals, 0.55 g (72.3%) yield, mp (°C)—138–139. IR (KBr): 3462, 3302, 3222 (NH_2+NH), 1650 (NCO) cm^{-1}. ^1H NMR (500 Mz: DMSO-d_6): δ 2.36 (s, 3H, CH_3), 3.84 (s, 2H, CH_2), 5.84 (s, 2H, NH_2, D_2O exchangeable), 7.18–7.32 (m, 10H, Ar-H), 12.16 (s, 1H, NH, D_2O exchangeable). Anal. Calcd for $C_{18}H_{18}N_6O$: C, 64.66; H, 5.43; N, 25.13. Found: C, 64.55; H, 5.64; N, 25.35.

5.3.12. 4-Amino-6-(4-methoxybenzyl)-3-(2-(1-phenyl-ethylidene)hydrazinyl)-1,2,4-triazin-5(4*H*)-one (2l)

Compound **2l** was obtained as yellow crystals, 0.62 g (71.2%) yield, mp (°C)—88–89. IR (KBr): 3406, 3304, 3218 (NH_2+NH), 1659 (NCO) cm^{-1}. ^1H NMR (500 MHz: DMSO-d_6): δ 2.36 (s, 3H, CH_3), 3.67 (s, 3H, OCH_3), 3.76 (s, 2H, CH_2), 5.85 (s, 2H, NH_2, D_2O exchangeable), 6.78–6.80 (m, 2H, Ar-H), 7.17 (d, 2H, J = 8.4 Hz, Ar-H), 7.36 (m, 3H, Ar-H), 7.75 (d, 2H, J = 6.1 Hz, Ar-H), 12.16 (s, 1H, NH, D_2O exchangeable). Anal. Calcd for $C_{19}H_{20}N_6O_2$: C, 62.62; H, 5.53; N, 23.06. Found: C, 62.50; H, 5.75; N, 22.87.

5.3.13. 4-Amino-6-benzyl-3-(2-(1-(4-chlorophenyl)ethylidene)-hydrazinyl)-1,2,4-triazin-5(4*H*)-one (2m)

Compound **2m** was obtained as yellow crystals, 0.66 g (78.5%) yield, mp (°C)—140–141. IR (KBr): 3462, 3328, 3241 (NH_2+NH), 1650 (NCO) cm^{-1}. ^1H NMR (500 MHz: DMSO-d_6): δ 2.34 (s, 3H, CH_3), 3.84 (s, 2H, CH_2), 5.85 (s, 2H, NH_2, D_2O exchangeable), 7.18–7.25 (m, 5H, Ar-H), 7.38 (d, 2H, J = 8.4 Hz, Ar-H), 8.08 (d, 2H, J = 8.4 Hz, Ar-H), 12.23 (s, 1H, NH, exchangeable). Anal. Calcd for $C_{18}H_{17}ClN_6O$: C, 58.62; H, 4.65; N, 22.79. Found: C, 58.86; H, 4.59; N, 22.53.

5.3.14. 4-Amino-3-(2-(1-(4-chlorophenyl)ethylidene)-hydrazinyl)-6-(4-methoxybenzyl)-1,2,4-triazin-5(4*H*)-one (2n)

Compound **2n** was obtained as yellow crystals, 0.68 g (70.8%) yield, mp (°C)—168–169. IR (KBr): 3267, 3227, 3190 (NH_2+NH), 1675 (NCO) cm^{-1}. ^1H NMR (500 MHz: DMSO-d_6): δ 2.34 (s, 3H, CH_3), 3.67 (s, 3H, OCH_3), 3.80 (s, 2H, CH_2), 5.55 (s, 2H, NH_2, D_2O exchangeable), 6.79 (d, 2H, J = 8.4 Hz, Ar-H), 6.81 (d, 2H, J = 8.4 Hz, Ar-H), 7.12(d, 2H, J = 8.4 Hz, Ar-H), 7.15 (d, 2H, J = 8.4 Hz, Ar-H), 12.51 (br s, 1H, NH, D_2O exchangeable). Anal. Calcd for $C_{19}H_{19}ClN_6O_2$: C, 57.22; H, 4.80; N, 21.07. Found: C, 57.05; H, 4.98; N, 21.30.

5.3.15. 4-Amino-6-zbenzyl-3-(2-(1-*p*-tolylethylidene)-hydrazinyl)-1,2,4-triazin-5(4*H*)-one (2o)

Compound **2o** was obtained as yellow crystals, 0.65 g (83.3%) yield, mp (°C)—146–147. IR (KBr): 3460, 3326, 3240 (NH_2+NH), 1650 (NCO) cm^{-1}. ^1H NMR (500 MHz: DMSO-d_6): δ 2.30 (s, 3H, CH_3), 2.33 (s, 3H, CH_3), 3.83 (s, 2H, CH_2), 5.83 (s, 2H, NH_2, D_2O exchangeable), 7.13–7.26 (m, 7H, Ar-H), 7.94 (d, 2H, J = 7.6 Hz, Ar-H), 12.11 (br s, 1H, NH, exchangeable). Anal. Calcd for $C_{19}H_{20}N_6O$: C, 65.50; H, 5.79; N, 24.12. Found: C, 65.63; H, 5.91; N, 23.82.

5.3.16. 4-Amino-6-(4-methoxybenzyl)-3-(2-(1-*p*-tolylethylidene)hydrazinyl)-1,2,4-triazin-5(4*H*)-one (2p)

Compound **2p** was obtained as yellow crystals, 0.70 g (73.6%) yield, mp (°C)—110–111. IR (KBr): 3462, 3334, 3235 (NH_2+NH), 1674 (NCO) cm^{-1}. ^1H NMR (500 MHz: DMSO-d_6): δ 2.21 (s, 3H, CH_3), 2.31 (s, 3H, CH_3), 3.67 (s, 3H, OCH_3), 3.76 (s, 2H, CH_2), 5.82 (s, 2H, NH_2, D_2O exchangeable), 6.81 (d, 2H, J = 8.4 Hz, Ar-H), 7.15–7.27 (m, 2H, Ar-H), 7.76 (d, 2H, J = 7.6 Hz, Ar-H), 7.93 (d, 2H, J = 7.6 Hz, Ar-H), 12.10 (s, 1H, NH). Anal. Calcd for $C_{20}H_{22}N_6O_2$: C, 63.48; H, 5.86; N, 22.21. Found: C, 63.20; H, 5.54; N, 22.04.

5.3.17. 4-Amino-6-benzyl-3-(2-(1-(pyridin-2-yl)ethylidene)-hydrazinyl)-1,2,4-triazin-5(4*H*)-one (2q)

Compound **2q** was obtained as yellow crystals, 0.79 g (95.1%) yield, mp (°C)—133–134. IR (KBr): 3420, 3300, 3215 (NH_2+NH), 1677 (NCO) cm^{-1}. ^1H NMR (500 MHz: DMSO-d_6): δ 2.40 (s, 3H, CH_3), 3.86 (s, 2H, CH_2), 5,88 (s, 2H, NH_2, D_2O exchangeable), 7.18–7.26 (m, 6H, Ar-H), 7.73 (br s, 1H, Py-H), 8.52 (br s, 1H, Py-H), 8.63 (br s, 1H, Py-H), 12.33 (s, 1H, NH, D_2O exchangeable). Anal. Calcd for $C_{17}H_{17}N_7O$: C, 60.88; H, 5.11; N, 29.24. Found: C, 60.69; H, 5.38; N, 29.51.

5.3.18. 4-Amino-6-(4-methoxybenzyl)-3-(2-(1-(pyridin-2-yl)ethylidene)hydrazinyl)-1,2,4-triazin-5(4*H*)-one (2r)

Compound **2r** was obtained as yellow crystals, 0.87 g (95.5%) yield, mp (°C)—246–247. IR (KBr): 3322, 3187 (NH_2+NH), 1685 (NCO) cm^{-1}. ^1H NMR (500 MHz: DMSO-d_6): δ 2.45 (s, 3H, CH_3), 3.68 (s, 3H, OCH_3), 3.81 (s, 2H, CH_2), 5.76 (s, 2H, NH_2, D_2O exchangeable), 6.80 (d, 2H, J = 8.4 Hz, Ar-H), 7.13 (d, 2H, J = 8.4 Hz, Ar-H), 7.84 (m, 1H, Py-H), 8.26 (d, 1H, J = 6.1 Hz, Py-H), 8.43 (m, 1H, Py-H), 8.79–8.82 (m, 1H, Py-H), 13.14 (s, 1H, NH, D_2O exchangeable). Anal. Calcd for $C_{18}H_{19}N_7O_2$: C, 59.17; H, 5.24; N, 26.83. Found: C, 58.92; H, 5.47; N, 26.73.

5.3.19. 4-Amino-6-benzyl-3-(2-(1-(furan-2-yl)ethylidene)-hydrazinyl)-1,2,4-triazin-5(4*H*)-one (2s)

Compound **2s** was obtained as brown crystals, 0.78 g (96.2%) yield, mp (°C)—69–70. IR (KBr): 3402, 3310, 3128 (NH_2+NH), 1684 (NCO) cm^{-1}. ^1H NMR (500 MHz: DMSO-d_6): δ 2.26 (s, 3H, CH_3), 3.83 (s, 2H, CH_2), 5.83 (s, 2H, NH_2, D_2O exchangeable), 6.55 (m, 1H, Fur-H_4), 7.20–7.26 (m, 6H, Ar-H), 7.72 (br s, 1H, Fur-H_5), 12.06 (s, 1H, NH, D_2O exchangeable). Anal. Calcd for $C_{16}H_{16}N_6O_2$: C, 59.25; H, 4.97; N, 25.91. Found: C, 59.13; H, 5.10; N, 26.10.

5.3.20. 4-Amino-3-(2-(1-(furan-2-yl)ethylidene)hydrazinyl)-6-(4-methoxybenzyl)-1,2,4-triazin-5(4*H*)-one (2t)

Compound **2t** was obtained as brown crystals, 0.81 g (92.0%) yield, mp (°C)—84–85. IR (KBr): 3399, 3309, 3200 (NH_2+NH), 1638 (NCO) cm^{-1}. ^1H NMR (500 MHz: DMSO-d_6) δ 2.25 (s, 3H, CH_3), 3.67 (s, 3H, CH_3), 3.75 (s, 2H, CH_2), 5.82 (s, 2H, NH_2, D_2O exchangeable), 6.54 (m, 1H, Fur-H_4), 6.81 (d, 2H, J = 7.7 Hz, Ar-H), 7.14 (d, 2H, J = 7.7 Hz, Ar-H), 7.20 (d, 1H, J = 3.1 Hz, Fur-H_3), 7.72 (br s, 1H, Fur-H_5) 12.01 (s, 1H, NH, D_2O exchangeable. Anal. Calcd for $C_{17}H_{18}N_6O_3$: C, 57.62; H, 5.12; N, 23.72. Found: C, 57.85; H, 4.86; N, 23.50.

5.4. General procedure for the reaction of Schiff's bases with acetic anhydride

A solution of the corresponding Schiff's base (2.5 mmol) in 6 mL acetic anhydride was heated under reflux for 10 h. The reaction mixture was then cooled and poured into crushed ice. The crude product was obtained by filtration, washed well with water, and recrystallized from ethanol.

5.4.1. 2-Acetyl-8-(*N*,*N*-diacetylamino)-6-benzyl-7-oxo-3-phenyl-2,3-dihydro-7*H*-[1,2,4] triazolo[4,3-*b*][1,2,4]triazine (3a)

Compound **3a** was obtained as brown powder, 0.95 g (94.0%) yield, mp (°C)—60–61. IR (KBr): 1755, 1708, 1699 (NCO) cm^{-1}. ^1H NMR (500 MHz: DMSO-d_6): δ 1.87 (s, 3H, CH_3), 2.02 (s, 3H, CH_3), 2.07 (s, 3H, CH_3), 4.01 (s, 2H, CH_2), 7.33–7.40 (m, 9H, Ar-H+CH), 7.45–7.46 (m, 1H, Ar-H). ^{13}C NMR (125 MHz: DMSO-d_6): δ 21.01, 21.24, 21.59, 37.32, 89.61, 128.83, 129.28, 129.32, 129.81, 146.67, 149.89, 154.73, 167.74, 169.27, 170.02. Anal. Calcd for $C_{23}H_{22}N_6O_4$: C, 61.87; H, 4.97; N, 18.82. Found: C, 61.48; H, 5.23; N, 19.02.

5.4.2. 2-Acetyl-8-(*N*,*N*-diacetylamino)-6-(4-methoxybenzyl)-7-oxo-3-phenyl-2,3-dihydro-7*H*-[1,2,4]triazolo[4,3-*b*][1,2,4]triazine (3b)

Compound **3b** was obtained as yellow crystals, 0.96 g (83.4%) yield, mp (°C)—155–156. IR (KBr): 1738, 1645 (NCO) cm^{-1}. ^1H NMR (500 MHz: DMSO-d_6): δ 2.01 (s, 3H, CH_3), 2.36 (s, 3H, CH_3), 2.46 (s, 3H, CH_3), 3.66 (s, 5H, CH_2+OCH_3), 6.74 (d, 2H, J = 7.6 Hz, Ar-H), 6.96 (d, 2H, J = 8.4 Hz, Ar-H), 7.00 (s, 1H, CH), 7.41–7.42 (m, 5H, Ar-H). ^{13}C NMR (125 MHz: DMSO-d_6), HMQC: δ 21.04 (CH_3), 24.10 (CH_3), 25.03 (CH_3), 40.00 (OCH_3), 55.52 (CH_2), 79.61 (CH), 114.29 (Ar-CH), 127.74 (Ar-CH), 128.29 (Ar-CH), 130.04 (Ar-CH), 130.21 (Ar-C), 136.80 (Ar-C), 143.26 (Ar-C), 151.93 (Ar-C), 158.55 (C=O), 167.19 (C=O), 168.83 (C=O), 170.09 (C=O). Anal. Calcd for $C_{24}H_{24}N_6O_5$: C, 60.50; H, 5.08; N, 17.64. Found: C, 60.24; H, 5. 32; N, 17.45.

5.4.3. 2-Acetyl-8-(*N*,*N*-diacetylamino)-6-benzyl-3-(4-methoxyphenyl)-7-oxo-2,3-dihydro-7*H*-[1,2,4]triazolo[4,3-*b*][1,2,4]triazine (3c)

Compound 3c was obtained as yellow crystals, 0.95 g (87.9%) yield, mp (°C)—140–141. IR (KBr): 1741, 1641 (NCO) cm^{-1}. ^1H NMR (500 MHz: DMSO-d_6): δ 2.00 (s, 3H, CH_3), 2.35 (s, 3H, CH_3), 2.46 (s, 3H, CH_3), 3.74–3.76 (m, 5H, CH_2+OCH_3), 6.95 (d, 2H, J = 3.0 Hz, Ar-H), 6.97 (s, 1H, CH), 7.06 (d, 2H, J = 7.3 Hz, Ar-H), 7.14–7.22 (m, 3H, Ar-H), 7.31 (d, 2H, J = 8.4 Hz, Ar-H). ^{13}C NMR (125 MHz: DMSO-d_6): δ 21.08, 24.05, 25.06, 40.00, 55.80, 79.42, 114.60, 128.90, 129.21, 136.68, 142.85, 144.62, 152.00, 160.72, 167.07, 168.79, 170.14. Anal. Calcd for $C_{24}H_{24}N_6O_5$: C, 60.50; H, 5.08; N, 17.64; O. Found: C, 60.77; H, 4.87; N, 17.52.

5.4.4. 2-Acetyl-8-(*N*,*N*-diacetylamino)-6-(4-methoxybenzyl)-3-(4-methoxyphenyl)-7-oxo-2,3-dihydro-7*H*-[1,2,4]triazolo[4,3-*b*][1,2,4]triazine (3d)

Compound **3d** was obtained as pale brown crystals, 0.92 g (72.7%) yield, mp (°C)—170–171. UV/VIS (CH_3CN) λ_{max} = 201 nm, ε = 11850; λ_{max} = 229 nm, ε = 13210; λ_{max} = 258 nm, ε = 9820. IR (KBr) 1738, 1644 (C=O) cm^{-1}. ^1H NMR (500 MHz: DMSO-d_6): δ 1.99 (s, 3H, CH_3), 2.35 (s, 3H, CH_3), 2.46 (s, 3H, CH_3), 3.66 (s, 6H, OCH_3), 3.68 (s, 2H, CH_2), 6.75 (d, 2H, J = 8.4 Hz, Ar-H), 6.95–6.98 (m, 5H, Ar-H+CH), 7.31 (d, 2H, J = 8.4 Hz, Ar-H). ^{13}C NMR (125 MHz: DMSO-d_6): δ 21.09, 24.07, 25.07, 35.40, 55.50, 55.79, 79.38, 114.29, 114.60, 128.97, 129.20, 130.03, 143.14, 144.64, 151.96, 158.53, 160.71, 167.04, 168.78, 170.16. Anal. Calcd for $C_{25}H_{26}N_6O_6$: C, 59.28; H, 5.17; N, 16.59. Found: C, 59.56; H, 4. 95; N, 16.32.

5.4.5. 2-Acetyl-8-(*N*,*N*-diacetylamino)-3-(4-chlorophenyl)-6-(4-methoxybenzyl)-3-methyl-7-oxo-2,3-dihydro-7*H*-[1,2,4]triazolo[4,3-*b*][1,2,4]triazine (3e)

Compound **3e** was obtained as pale yellow crystals, 0.78 g (65%) yield, mp (°C)—135–136. IR (KBr): 3439 (NH), 1730 (C=O) cm^{-1}. ^1H NMR (500 MHz: DMSO-d_6): δ 2.36 (s, 6H, 2CH_3), 2.50 (s, 3H, CH_3), 3.68 (s, 3H, OCH_3), 4.03 (s, 2H, CH_2), 6.82 (s, 1H, NH, D_2O exchangeable), 6.84 (d, 4H, J = 8.4 Hz, Ar-H), 7.23 (d, 4H, J = 8.4 Hz, Ar-H). Anal. Calcd for $C_{25}H_{25}ClN_6O_5$: C, 57.20; H, 4.80; N, 16.01. Found: C, 57.02; H, 4.57; N, 15.79.

5.4.6. 2-Acetyl-8-(*N*,*N*-diacetylamino)-6-(4-methoxybenzyl)-3-methyl-7-oxo-3-*p*-tolyl-2,3-dihydro-7*H*-[1,2,4]triazolo[4,3-*b*][1,2,4]triazine (3f)

Compound **3f** was obtained as dark orange crystals, 0.97 g (76.9%) yield, mp (°C)—140–141. IR (KBr): 1740, 1649 (C=O) cm^{-1}. ^1H NMR (500 MHz: DMSO-d_6): δ 1.99 (s, 3H, CH_3), 2.19 (s, 3H, CH_3), 2.28 (s, 3H, CH_3), 2.38 (s, 3H, CH_3), 2.43 (s, 3H, CH_3), 3.64 (d, 5H, J = 10.0 Hz, CH_2+OCH_3), 6.74 (d, 2H, J = 8.4 Hz, Ar-H), 6.92 (d, 2H, J = 8.4 Hz, Ar-H), 7.20 (d, 2H, J = 8.4 Hz, Ar-H), 7.24 (d, 2H, J = 8.4 Hz, Ar-H). ^{13}C NMR (125 MHz: DMSO-d_6): .δ 21.18, 22.04, 22.12, 24.27, 24.90, 35.38, 55.50, 87.00, 114.28, 126.52, 139.53, 130.05, 136.26, 139.01, 143.08, 143.41, 152.07, 158.53, 166.04, 168.90, 170.03. Anal. Calcd for $C_{26}H_{28}N_6O_5$: C, 61.89; H, 5.59; N, 16.66. Found: C, 62.06; H, 5. 32; N, 16.53.

5.4.7. 2-Acetyl-8-(*N*-acetylamino)-6-benzyl-3-(4-nitrophenyl)-7-oxo-2,3-dihydro-7*H*-[1,2,4]triazolo[4,3-*b*][1,2,4]triazine (4a)

Compound **4a** was obtained as brown crystals, 0.96 g (85.7%) yield, mp (°C)—242–243. IR (KBr): 3193 (NH), 1727, 1706 (C=O) $cm^{-1.}$ ^1H NMR (500 MHz: DMSO-d_6): (**Isomer 1**, **57**%) δ 2.00(s, 3H, CH_3), δ 2.02(s, 3H, CH_3), 3.62–3.76 (m, 2H, CH_2), 7.09–7.20 (m, 6H, Ar-H+CH), 7.64 (d, 1H, J = 8.4 Hz), 7.75 (d, 1H, J = 8.4 Hz, Ar-H), 8.24–8.28 (m, 2H, Ar-H), 11.14 (d, 1H, NH, D_2O exchangeable). (**Isomer 2**, **43**%) δ 2.00 (s, 3H, CH_3), δ 2.05(s, 3H, CH_3), 3.62–3.76 (m, 2H, CH_2), 7.09–7.20 (m, 6H, Ar-H+CH), 7.64 (d, 1H, J = 8.4 Hz), 7.75 (d, 1H, J = 8.4 Hz, Ar-H), 8.24–8.28 (m, 2H, Ar-H), 11.14 (d, 1H, NH, D_2O exchangeable). ^{13}C NMR (125 MHz: DMSO-d_6): δ 20.73, 20.95, 39.66, 78.22, 129.13, 129.16, 136.59, 136.66, 143.22, 145.91, 152.25, 161.36, 167.28, 168.70. Anal. Calcd for $C_{21}H_{19}N_7O_5$: C, 56.12; H, 4.26; N, 21.82. Found: C, 55.84; H, 4.52; N, 22.06.

5.4.8. 2-Acetyl-8-(*N*-acetylamino)-6-(4-methoxybenzyl)-3-(4-nitrophenyl)-7-oxo-2,3-dihydro-7*H*-[1,2,4]triazolo[4,3-*b*][1,2,4]triazine (4b)

Compound **4b** was obtained as dark brown crystals, 0.89 g (68.4%) yield, mp (°C)—120–121. IR (KBr): 3465 (NH), 1744, 1656 (C=O) cm^{-1}. ^1H NMR (500 Hz: DMSO): δ 1.99–2.04 (m, 6H, CH_3), 3.36 (s, 3H, OCH_3), 3.65 (s, 2H, CH_2), 6.74 (d, 2H, J = 8.4 Hz, Ar-H), 6.97–7.01 (m, 2H, Ar-H), 7.15 (s, 1H, CH), 7.69 (d, 1H, J = 7.6 Hz, Ar-H), 8.24 (d, 3H, J = 5.3 Hz, Ar-H), 11.03 (br s, 1H, NH, D_2O exchangeable). Anal. Calcd for $C_{22}H_{21}N_7O_6$: C, 55.11; H, 4.41; N, 20.45. Found: C, 55.38; H, 4.14; N, 20.72.

5.4.9. 2-Acetyl-8-(*N*-acetylamino)-6-benzyl-3-methyl-7-oxo-3-phenyl-2,3-dihydro-7*H*-[1,2,4]triazolo[4,3-*b*][1,2,4]triazine (4c)

Compound **4c** was obtained as brown crystals, 0.85 g (81.7%) yield, mp (°C)—58–59. IR (KBr): 3455 (NH), 1738, 1649 (C=O) cm^{-1}. ^1H NMR (500 MHz: DMSO-d_6): (**Isomer 1**, **26**%) δ 2.01 (s, 3H, CH_3), 2.17 (s, 3H, CH_3), 2.36 (s, 3H, CH_3), 3.67 (s, 2H, CH_2), 7.00–7.04 (m, 2H, Ar-H), 7.15–7.21 (m, 3H, Ar-H), 7.37–7.41 (m, 5H, Ar-H), 11.04 (s, 1H, NH, D_2O exchangeable). (**Isomer 2**, **74**%) δ 1.99 (s, 3H, CH_3), 2.22 (s, 3H, CH_3), 2.39 (s, 3H, CH_3), 3.73 (s, 2H, CH_2), 7.00–7.04 (m, 2H, Ar-H), 7.15–7.21 (m, 3H, Ar-H), 7.37–7.41 (m, 5H, Ar-H), 11.04 (s, 1H, NH, D_2O exchangeable). ^{13}C NMR (125 MHz: DMSO-d_6): δ 22.11, 24.26, 24.92, 39.822, 87.01, 128.89, 128.95, 129.02, 142.93, 143.47, 152.10, 168.42, 168.90, 170.07. Anal. Calcd for $C_{22}H_{22}N_6O_3$: C, 63.15; H, 5.30; N, 20.08. Found: C, 63.02; H, 5.52; N, 20.18.

5.4.10. 2-Acetyl-8-(*N*-acetylamino)-6-(4-methoxybenzyl)-3-methyl-7-oxo-3-phenyl-2,3-dihydro-7*H*-[1,2,4]triazolo[4,3-*b*][1,2,4]triazine (4d)

Compound **4d** was obtained as orange crystals, 0.95 g (77.8%) yield, mp (°C)—103–104. IR (KBr): 3449 (NH), 1707, 1638 (C=O) cm^{-1}. ^1H NMR (500 MHz: DMSO-d_6): (**Isomer 1**, **47**%) δ 1.93 (s, 3H, CH_3), 2.01 (s, 3H, CH_3), 2.16 (s, 3H, CH_3), 3.57 (s, 2H, CH_2), 3.66 (s, 3H, OCH_3), 6.74 (d, 2H, J = 8.4 Hz, Ar-H), 6.94 (t, 2H, J = 7.6 Hz, Ar-H), 7.37–7.42 (m, 5H, Ar-H), 12.23 (s, 1H, NH, D_2O, exchangeable). (**Isomer 2**, **53**%) δ 1.94 (s, 3H, CH_3), 2.00 (s, 3H, CH_3), 2.19 (s, 3H, CH_3), 3.59 (s, 2H, CH_2), 3.66 (s, 3H, OCH_3), 6.74 (d, 2H, J = 8.4 Hz, Ar-H), 6.94 (t, 2H, J = 7.6 Hz, Ar-H), 7.37–7.42 (m, 5H, Ar-H), 12.23 (s, 1H, NH, D_2O, exchangeable). ^{13}C NMR (125 MHz: DMSO-d_6): δ 21.95, 22.18, 22.22, 35.43, 55.53, 86.06, 114.25, 126.40, 128.98, 129.34, 130.21, 139.44, 143.14, 144.62,

152.09, 152.34, 158.52, 165.85, 168.75. Anal. Calcd for $C_{23}H_{24}N_6O_4$: C, 61.60; H, 5.39; N, 18.74. Found: C, 61.63; H, 5.37; N, 18.76.

5.4.11. 2-Acetyl-8-(*N*-acetylamino)-6-benzyl-3-(4-chlorophenyl)-3-methyl-7-oxo-2,3-dihydro-7*H*-[1,2,4]triazolo[4,3-*b*][1,2,4]triazine (4e)

Compound **4e** was obtained as orange crystals, 1.00 g (81.3%) yield, mp (°C)—210–211. IR (KBr): 3448 (NH), 1717, 1634 (C=O) cm^{-1}. ^1H NMR (500 MHz: DMSO-d_6): (**Isomer 1**, **50**%) δ 1.98 (s, 6H, CH_3), 2.13 (s, 3H, CH_3), 3.66–3.72 (m, 2H, CH_2), 7.06 (d, 2H, J = 7.6 Hz, Ar-H), 7.15–7.21 (m, 3H, Ar-H), 7.38 (d, 1H, J = 6.8 Hz, Ar-H), 7.43–7.48 (m, 3H, Ar-H), 10.46 (br s, 1H, NH, D_2O exchangeable). (**Isomer 2**, **50**%) δ 2.01 (s, 6H, CH_3), 2.18 (s, 3H, CH_3), 3.66–3.72 (m, 2H, CH_2), 7.06 (d, 2H, J = 7.6 Hz, Ar-H), 7.15–7.21 (m, 3H, Ar-H), 7.38 (d, 1H, J = 6.8 Hz, Ar-H), 7.43–7.48 (m, 3H, Ar-H), 10.46 (br s, 1H, NH, D_2O exchangeable). ^{13}C NMR (125 MHz: DMSO-d_6): δ 20.79, 21.84, 22.124, 36.22, 85.85, 127.22, 128.43, 128.89, 129.09, 129.16, 136.69, 152.12, 152.33, 166.04, 166.07, 168.52. Anal. Calcd for $C_{22}H_{21}ClN_6O_3$: C, 58.34; H, 4.67; N, 18.56. Found: C, 58.58; H, 4.83; N, 18.25.

5.4.12. 2-Acetyl-8-(*N*-acetylamino)-6-benzyl-3-methyl-7-oxo-3-p-tolyl-2,3-dihydro-7*H*-[1,2,4]triazolo[4,3-*b*][1,2,4]triazine (4f)

Compound **4f** was obtained as orange crystals, 0.95 g (80.5%) yield, mp (°C)—194–195. IR (KBr): 3409 (NH), 1702 (C=O) cm^{-1}. ^1H NMR (500 MHz: DMSO-d_6): (**Isomer 1**, **54**%) δ 1.98 (s, 3H, CH_3), 2.00 (s, 3H, CH_3), 2.01 (s, 3H, CH_3), 2.27 (s, 3H, CH_3), 3.65–3.75 (m, 2H, CH_2), 7.04–7.06 (m, 2H, Ar-H), 7.15–7.28 (m, 6H, Ar-H), 7.30 (d, 1H, J = 7.6 Hz, Ar-H), 12.21 (s, 1H, NH, D_2O exchangeable). (**Isomer 2**, **46**%) δ 1.98 (s, 3H, CH_3), 2.01 (s, 3H, CH_3), 2.15 (s, 3H, CH_3), 2.28 (s, 3H, CH_3), 3.65–3.75 (m, 2H, CH_2), 7.04–7.06 (m, 2H, Ar-H), 7.15–7.28 (m, 6H, Ar-H), 7.30 (d, 1H, J = 7.6 Hz, Ar-H), 12.21 (s, 1H, NH, D_2O exchangeable). ^{13}C NMR (125 MHz: DMSO-d_6): δ 20.79, 21.18, 22.00, 22.22, 36.27, 86.39, 126.34, 126.62, 127.15, 128.90, 136.22, 136.58, 138.86, 142.65, 144.54, 152.38, 165.82, 165.91, 168.43. Anal. Calcd for $C_{23}H_{24}N_6O_3$: C, 63.88; H, 5.59; N, 19.43. Found: C, 63.65; H, 5.71; N, 19.73.

5.4.13. 2-Acetyl-8-(*N*-acetylamino)-6-benzyl-3-methyl-7-oxo-3-(pyridin-2-yl)-2,3-dihydro-7*H*-[1,2,4]triazolo[4,3-*b*][1,2,4]triazine (4g)

Compound **4g** was obtained as orange crystals, 0.94 g (91.3%) yield, mp (°C)—280–281. IR (KBr): 3200 (NH), 1713, 1672 (C=O) cm^{-1}. ^1H NMR (500 MHz: DMSO-d_6): (**Isomer 1**, **45**%) δ 1.96 (s, 3H, CH_3), 2.21(s, 3H, CH_3), 2.23 (d, 3H, J = 13 Hz, CH_3), 3.59–3.72 (m, 2H, CH_2), 6.94 (d, 1H, J = 7.6 Hz, Ar-H), 7.01 (d, 1H, J = 7.6 Hz, Ar-H), 7.14–7.18 (m, 3H, Ar-H), 7.37–7.40 (m, 1H, Ar-H), 7.65–7.66 (m, 1H, Ar-H), 7.82–7.87 (m, 1H, Ar-H), 8.53–8.56 (m, 1H, Ar-H), 11.10 (s, 1H, NH, D_2O exchangeable). (**Isomer2**, **55**%) δ 1.97 (s, 3H, CH_3), 2.23 (s, 3H, CH_3), 2.23 (d, 3H, J = 13 Hz, CH_3), 3.59–3.72 (m, 2H, CH_2), 6.94 (d, 1H, J = 7.6 Hz, Ar-H), 7.01 (d, 1H, J = 7.6 Hz, Ar-H), 7.14–7.18 (m, 3H, Ar-H), 7.37–7.40 (m, 1H, Ar-H), 7.65–7.66 (m, 1H, Ar-H), 7.82–7.87 (m, 1H, Ar-H), 8.53–8.56 (m, 1H, Ar-H), 11.10 (s, 1H, NH, D_2O exchangeable). ^{13}C NMR (125 MHz: DMSO-d_6): δ 20.88, 21.02, 21.33, 39.96, 86.62, 122.16, 124.94, 128.78, 129.10, 136.70, 136.82, 142.80, 144.81, 149.50, 152.00, 156.79, 166.19, 168.36, 168.49. Anal. Calcd for $C_{21}H_{21}N_7O_3$: C, 60.13; H, 5.05; N, 23.38. Found: C, 60.18; H, 5.30; N, 23.26.

5.4.14. 2-Acetyl-8-(*N*-acetylamino)-6-benzyl-3-(furan-2-yl)-3-methyl-7-oxo-2,3-dihydro-7*H*-[1,2,4]triazolo[4,3-*b*][1,2,4]triazine (4h)

Compound **4h** was obtained as yellow crystals, 0.89 g (87.2%) yield, mp (°C)—220–221. IR (KBr): 3177 (NH), 1741, 1664 (C=O) cm^{-1}. ^1H NMR (500 MHz: DMSO): δ 1.97 (s, 3H, CH_3), 1.98 (s, 3H, CH_3), 2.12 (s, 3H, CH_3), 3.70 (s, 2H, CH_2), 6.44 (d, 1H, J = 8 Hz, Ar-H), 6.63–6.70 (m, 1H, Ar-H), 7.04–7.08 (m, 2H, Ar-H), 7.10–7.22 (m, 3H, Ar-H), 7.64 (s, 1H, Ar-H), 11.01 (s, 1H, NH, D_2O exchangeable). ^{13}C NMR (125 Hz: DMSO): δ 20.71, 20.89, 22.05, 31.32, 81.78, 110.89, 111.17, 128.83, 129.12, 129.17, 143.00, 144.24, 151.93, 152.26, 166.40, 168.41. Anal. Calcd for $C_{20}H_{20}N_6O_4$: C, 58.82; H, 4.94; N, 20.58. Found: C, 59.01; H, 4.69; N, 20.71.

5.4.15. 2-Acetyl-8-(*N*-acetylamino)-3-(furan-2-yl)-6-(4-methoxybenzyl)-3-methyl-7-oxo-2,3-7*H*-dihydro-[1,2,4]triazolo[4,3-*b*][1,2,4]triazine (4i)

Compound **4i** was obtained as pale yellow crystals, 0.83 g (76.1%) yield, mp (°C)—218–219 °C. IR (KBr): 3202 (NH), 1745, 1656 (C=O) cm^{-1}. ^1H NMR (500 MHz: DMSO-d_6): δ 1.97 (s, 3H, CH_3), 2.14 (s, 3H, CH_3), 2.40 (s, 3H, CH_3), 3.66 (brs, 5H, CH_2+OCH_3), 6.47–6.48 (m, 1H, Ar-H), 6.67 (d, 1H, J = 3.8 Hz, Ar-H), 6.77–6.79 (m, 2H, Ar-H), 6.94–7.00 (m, 2H, Ar-H), 7.64 (d, 1H, J = 11.4 Hz, Ar-H), 12.26 (s, 1H, NH, D_2O exchangeable). Anal. Calcd for $C_{21}H_{22}N_6O_5$: C, 57.53; H, 5.06; N, 19.17. Found: C, 57.84; H, 4.86; N, 18.99.

5.4.16. 2-Acetyl-8-(*N*-acetylamino)-6-benzyl-3-(4-methoxyphenyl)-7-oxo-2,3-dihydro-7*H*-[1,2,4]triazolo[4,3-*b*][1,2,4]triazine (4j)

To a solution of *N*-acetyl-*N*-(2-acetyl-6-benzyl-2,3-dihydro-3-(4-methoxyphenyl)-7-oxo-[1,2,4]triazolo[4,3-*b*][1,2,4]triazin-8(7*H*)-yl)acetamide **3c** (0.48 g, 1 mmol) in 2 ml ethanol, a solution of o-phenylene diamine (0.1 g, 1 mmol) in 1 ml ethanol was added. The reaction mixture was then heated under reflux for about 3 h. The product was collected from the cooled reaction mixture by filtration and recrystallized from ethanol to afford compound **4j** as brown crystals, 0.39 g (92.8%) yield, mp (°C)—252–253. IR (KBr): 3146 (NH), 1730, 1701 (C=O) cm^{-1}. ^1H NMR (500 MHz: DMSO-d_6): δ 1.99 (s, 3H, CH_3), 2.02 (s, H, $3CH_3$), 3.61–3.74 (m, 5H, CH_2+OCH_3), 6,88 (s, 1H, CH), 6.94–6.96 (m, 2H, Ar-H), 7.08 (d, 2H, J = 6.9 Hz, Ar-H), 7.15–7.20 (m, 3H, Ar-H), 7.27 (d, 1H, J = 8.4 Hz, Ar-H), 7.35 (d, 1H, J = 8.4 Hz, Ar-H), 11.17 (s, 1H, NH, D_2O exchangeable). ^{13}C NMR (125 MHz: DMSO-d_6): δ 20.72, 20.87, 39.19, 55.86, 79.14, 114.71, 128.83, 129.16, 136.60, 143.08, 145.63, 152.16, 160.61, 167.19, 169.14. EIMS (*m*/*z*): 434 (44, $[M]^+$), 91 (100). Anal. Calcd for $C_{22}H_{22}N_6O_4$: C, 60.82; H, 5.10; N, 19.34. Found: C, 61.05; H, 4.98; N, 19.74.

5.5. General procedure for the reaction of 4-amino-6-aryl-3-hydrazinyl-1,2,4-triazin-5(4*H*)-one (1) with isatine (5)

A mixture of 4-amino-6-aryl-3-hydrazinyl-1,2,4-triazin-5(4*H*)-one (**1**) (0.5 mmol) and isatine (0.07 g, 0.5 mmol) in 5 mL methanol was heated under refluxed for 2 h then cooled to room temperature. The product that separated out was then collected by filtration and recrystallized from ethanol.

5.5.1. 3-(2-(4-Amino-6-benzyl-5-oxo-4,5-dihydro-1,2,4-triazin-3-yl)hydrazono)indolin-2-one (5a)

Compound **5a** was obtained as yellow powder, 0.12 g (79.8%) yield, mp (°C)—250–251. IR (KBr): 3408, 3308 (NH_2+NH), 1700 (NCO) cm^{-1}. ^1H NMR (500 MHz: DMSO-d_6): δ 3.90 (s, 2H, CH_2), 6.02 (s, 2H, NH_2, D_2O exchangeable), 6.83–6.86 (m, 1H, Ar-H), 6.97–7.00 (m, 1H, Ar-H), 7.18–7.31 (m, 6H, Ar-H), 8.33–8.37 (m, 2H, Ar-H+NH, D_2O exchangeable), 10.63 (s, 1H, NH, D_2O exchangeable). ^{13}C NMR (125 MHz: DMSO-d_6): 36.35, 110.39, 127.07, 128.19, 129.46, 131.84, 137.37, 143.37, 145.08, 150.88, 151.11, 166.18. Anal. Calcd for $C_{18}H_{15}N_7O_2$: C, 59.83; H, 4.18; N, 27.13. Found: C, 59.55; H, 4.44; N, 27.33.

5.5.2. 3-(2-(4-Amino-6-(4-methoxybenzyl)-5-oxo-4,5-dihydro-1,2,4-triazin-3-yl)hydrazono) indolin-2-one (5b)

Compound **5b** was obtained as yellow crystals, 0.1 g (47.6%) yield, mp (°C)—235–236. IR (KBr): 3431, 3302 (NH_2+NH), 1701 (NCO) cm^{-1}. ^1H NMR (500 MHz: DMSO-d_6): δ 3.67 (s, 3H, OCH_3), 3.81 (s, 2H, CH_2), 6.00 (s, 2H, NH_2, D_2O exchangeable), 6.82 (d, 2H, J = 8.4 Hz, Ar-H), 6.94 (t, 1H, J = 7.6 Hz, Ar-H), 7.16–7.24 (m, 4H, Ar-H), 8.31–8.32 (m, 3H, Ar-H+NH, D_2O exchangeable), 10.56 (s, 1H, NH, D_2O exchangeable). Anal. Calcd for $C_{19}H_{17}N_7O_3$: C, 58.31; H, 4.38; N, 25.05. Found: C, 59.05; H, 4.68; N, 25.15.

5.6. General procedure for the preparation of 8-(*N*,*N*-diacetylamino)-1′,2-diacetyl-6-substituted benzyl-2*H*-spiro[1,2,4]triazolo[4,3-b][1,2,4]triazine-3,3′-indoline]-2′,7(8*H*)-dione (6)

A solution of 3-(2-(4-amino-6-aryl-5-oxo-4,5-dihydro-1,2,4-triazin-3-yl)hydrazono) indolin-2-one (**5**) (0.5 mmol) in 3 mL acetic anhydride was heated under reflux for 10 h. The reaction mixture was then cooled and poured into crushed ice. The resulting precipitate was filtered, washed with water, and recrystallized from ethanol.

5.6.1. 8-(*N*,*N*-Diacetylamino)-1′,2-diacetyl-6-benzyl-2*H*-spiro[[1,2,4]triazolo[4,3-b][1,2,4]triazine-3,3′-indoline]-2′,7(8*H*)-dione (6a)

Compound **6a** was collected as yellow crystals, 0.21 g (89.3%) yield, mp (°C)—165–166. IR (KBr): 1737, 1659 (NCO) cm^{-1}. ^1H NMR (500 MHz: DMSO-d_6): δ 2.06 (s, 3H, CH_3), 2.47 (s, 6H, $2CH_3$), 2.57 (s, 3H, CH_3), 3.74 (s, 2H, CH_2), 6.98 (d, 2H, J = 7.6 Hz, Ar-H), 7.13–7.20 (m, 3H, Ar-H), 7.34 (t, 1H, J = 16.0 Hz, Ar-H), 7.54–7.57 (m, 2H, Ar-H), 8.13 (d, 1H, J = 8.4 Hz, Ar-H).^{13}C NMR (125 MHz: DMSO-d_6): δ 20.51, 24.57, 24.68, 26.50, 36.13, 83.06, 122.45, 126.78, 127.30, 128.90, 133.09, 135.94, 141.07, 144.67, 146.04, 151.88, 167.14, 169.14, 170.27, 170.35. Anal. Calcd for $C_{26}H_{23}N_7O_6$: C, 58.98; H, 4.38; N, 18.52.Found: C, 59.75; H, 4.13; N, 18.44.

5.6.2. 8-(*N*,*N*-Diacetylamino)-1′,2-diacetyl-6-(4-methoxybenzyl)-2*H*-spiro[[1,2,4]triazolo [4,3-*b*][1,2,4]triazine-3,3′-indoline]-2′,7(8*H*)-dione (6b)

Compound **6b** was collected as yellow crystals, 0.13 g (85.5%) yield, mp (°C)—205–206. IR (KBr): 1746, 1655 (NCO) cm^{-1}. ^1H NMR (500 MHz: DMSO-d_6): δ 2.06 (s, 3H, CH_3), 2.45 (s, 3H, CH_3), 2.46 (s, 3H, CH_3), 2.57 (s, 3H, CH_3), 3.65 (s, 5H, CH_2+OCH_3), 6.74 (d, 2H, J = 8.4 Hz, Ar-H), 6.89 (d, 2H, J = 8.4 Hz, Ar-H), 7.37 (t, 1H, J = 7.6 Hz, Ar-H), 7.53–7.64 (m, 2H, Ar-H), 8.13 (d, 2H, J = 8.4 Hz, Ar-H). ^{13}C NMR (125 MHz: DMSO-d_6): δ 20.52, 24.55, 24.70, 26.51, 35.36, 55.55, 83.05, 114.31, 116.79, 125.33, 126.80, 127.58, 130.04, 133.09, 141.05, 144.66, 146.28, 151.84, 158.61, 167.14, 169.73, 170.27, 170.37. Anal. Calcd for $C_{27}H_{25}N_7O_7$: C, 57.96; H, 4.50; N, 17.52. Found: C, 57.77; H, 4.73; N, 17.65.

5.7. General procedure for the reaction of 4-amino-6-aryl-3-hydrazinyl-1,2,4-triazin-5(4*H*)-one (1) with triethylorhtoformate (9)

To a solution of 4-amino-6-aryl-3-hydrazinyl-1,2,4-triazin-5(4*H*)-one **1** (0.5 mmol) in 5 mL ethanol, triethyl orthoformate (0.1 mL, 0.5 mmol) and 2 drops of glacial acetic acid were added. The reaction mixture was then heated under reflux for about 10 h. The product was obtained on cooling, filtered and washed well with ethanol.

5.7.1 8-Amino-6-benzyl-[1,2,4]triazolo[4,3-*b*][1,2,4]triazin-7(8*H*)-one (9a)

Compound **9a** was obtained as colorless crystals, 0.08 g (66.6%) yield, mp (°C)—152–153. UV/VIS (CH_3CN): λ_{max} = 222 nm, ε = 10870; λ_{max} = 254 nm, ε = 7000; λ_{max} = 320 nm, ε = 2030. IR (KBr): 3321, 3220 (NH_2), 1689 (NCO) cm^{-1}; ^1H NMR (500 MHz: DMSO-d_6): δ 4.02 (s, 2H, CH_2), 5.94 (s, 2H, NH_2, D_2O exchangeable), 7.20–7.29 (m, 5H, Ar-H), 9.7 (s, 1H, CH). ^{13}C NMR (125 MHz: DMSO-d_6): δ 37.05, 127.33, 128.92, 129.93, 135.93, 138.91, 145.28, 152.18, 153.73. Anal. Calcd for $C_{11}H_{10}N_6O$: C, 54.54; H, 4.16; N, 34.69. Found: C, 54.32; H, 4.47; N, 34.39.

5.7.2. 8-Amino-6-(4-methoxybenzyl)-[1,2,4]triazolo[4,3-*b*][1,2,4]triazin-7(8*H*)-one (9b)

Compound **9b** was obtained as yellow crystals, 0.09 g (65.6%) yield, mp (°C)—272–273. IR (KBr): 3292, 3241 (NH_2), 1687 (NCO) cm^{-1}; ^1H NMR (500 MHz: DMSO-d_6): δ 3.67 (s, 3H, OCH_3), 3.70 (s, 2H, CH_2), 6.03 (s, 2H, NH_2, D_2O exchangeable), 6.80 (d, 2H, J = 7.6 Hz, Ar-H), 7.12 (d, 2H, J = 8.6 Hz, Ar-H), 11.91 (s, 1H, CH). ^{13}C NMR (125 MHz: DMSO-d_6): δ 39.84, 55.55, 114.22, 129.66, 130.58, 141.31, 143.09, 150.02, 158.41. Anal. Calcd for $C_{12}H_{12}N_6O_2$: C, 52.94; H, 4.44; N, 30.87. Found: C, 52.67; H, 4.40; N, 30.93.

5.8. General procedure for the reaction of 4-amino-6-aryl-3-hydrazinyl-1,2,4-triazin-5(4*H*)-one (1) with acetic anhydride (11)

A solution of 4-amino-6-aryl-3-hydrazinyl-1,2,4-triazin-5(4*H*)-one **1** (2.5 mmol) in 6 mL acetic anhydride was heated under reflux for 6 h. The reaction mixture was then cooled and poured into crushed ice. The resulting precipitate was filtered, washed with water, and recrystallized from ethanol.

5.8.1. 4-Acetyl-7-benzyl-3-methyl-1*H*-[1,2,4]triazino[4,3-*b*][1,2,4,5]tetrazin-6(4*H*)-one (11a)

Compound **11a** was obtained as yellow crystals, 0.2 g (28.5%) yield, mp (°C)—80–81. UV/VIS (CH_3CN): λ_{max} = 210 nm, ε = 18500; λ_{max} = 256 nm, ε = 10430. IR (KBr): 3166 (NH), 1711 (NCO) cm^{-1}. ^1H NMR (500 MHz: DMSO-d_6), NOE: δ 2.03 (s, 3H, CH_3), 2.47 (s, 3H, CH_3), 3.99–4.11 (m, 2H, CH_2), 7.26–7.30 (m, 5H, Ar-H), 11.35 (s, 1H, NH, D_2O exchangeable). ^{13}C NMR (125 MHz: DMSO-d_6): δ 9.71, 20.79, 37.18, 127.47, 128.99, 129.73, 135.74, 145.06, 146.87, 151.36, 153.25, 168.56. Anal. Calcd for $C_{14}H_{14}N_6O_2$: C, 56.37; H, 4.73; N, 28.1. Found: C, 56.12; H, 4.99; N, 27.85.

5.8.2. 4-Acetyl-7-(4-methoxybenzyl)-3-methyl-1*H*-[1,2,4]triazino[4,3-*b*][1,2,4,5]tetrazin-6(4*H*)-one (11b)

Compound **11b** was obtained as brown crystals, 0.25 g (36.2%) yield, mp (°C)—65–66. IR (KBr): 3234 (NH), 1697 (NCO) cm^{-1}. ^1H NMR (500 MHz: DMSO-d_6): δ 2.01 (s, 3H, CH_3), 2.42 (s, 3H, CH_3), 3.76 (s, 5H, OCH_3+CH_2), 6.83 (d, 2H, J = 8.4 Hz, Ar-H), 7.24 (d, 2H, J = 8.4 Hz Ar-H), 11.34 (s, 1H, NH, D_2O exchangeable). ^{13}C NMR (125 MHz: DMSO-d_6): δ 11.32, 20.93, 36.36, 55.56, 114.40, 129.19, 130.86, 132.16, 151.32, 153.47, 158.78, 168.68, 168.75. Anal. Calcd for $C_{15}H_{16}N_6O_3$: C, 54.87; H, 4.91; N, 25.60. Found: C, 55.15; H, 4.62; N, 25.46.

5.9. Biology

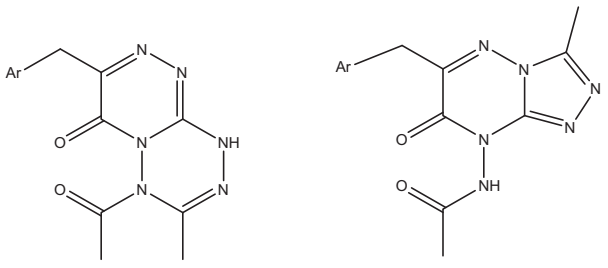
5.9.1. Microsomal AHH activity

Microsomal aryl hydrocarbon hydroxylase (AHH) activity was determined according to the method of Wiebel and Gelboin, 1975.[26] Briefly, the volume of the incubation mixture was 1 mL containing 50 mM Tris–HCl buffer, pH 7.4; 3 mmol $MgCl_2$; 0.6 mmol NADPH; 100 nmol of each triazine derivatives; 0.1 mL

of microsomal protein (10 mg:mL). The reaction was started by the addition of 100 nmol benzo[α]pyrene then incubated at 37 °C for 10 min. The reaction was terminated by the addition of 1 mL acetone. The 3-hydroxy benzo[α]pyrene was extracted in 2 mL hexane. The fluorescence intensity was measured at excitation and emission wavelengths of 396 and 522 nm, respectively. Hepatic microsome was prepared from PAH induced rat liver tissues which dissected into very small pieces before homogenization in 3 vol of 0.1 M of potassium phosphate buffer, pH 7.4. After centrifugation at 12,000 g for 20 min at 4 °C, the supernatant fractions was recentrifuged at 105,000 g for 1 h at 4 °C to yield a microsomal pellet which was then re-suspended in 0.1 M potassium phosphate buffer, pH 7.4.[27,28]

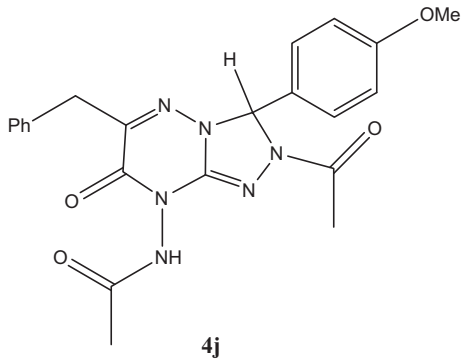
5.9.2. Oral and intraperitoneal acute toxicity tests

Oral and intraperitoneal acute toxicity tests were conducted with male mice (24 ± 1.9 g) obtained from Medical Research Institute, Alexandria University to determine the potential of test compounds **4e**, **5a**, **5b** and **6b** to produce toxicity. Animals were divided into groups of six mice each. Tested compounds were suspended in corn oil as a drug vehicle and given orally by oral gavage in doses of 1, 10, 100, 200 or 350 mg/kg or intraperitoneal in doses of 1, 5, 10, 50 or 150 mg/kg. Control group received corn oil only. Bodyweight were recorded prior to administration and again on day 7 and 14 (termination). The animals were observed for mortality, signs of gross toxicity and behavioral changes at least once daily and the percentage survival was recorded at termination.[29] The animal care local committee approved the design of the experiment, and the protocol conformed to the guidelines of the National Institutes of Health for animal care and handling.



11 **12**

	Ar
a	C_6H_5
b	4-OCH_3-C_6H_4



4j

5.10. Structural modeling and inhibitor docking

The 3D structure model of AHH (CYP1A1) was generated through comparative modeling using Geno 3D[37] and CYP1A2 (PDB: 2hi4) as a template. Triazolotriazine derivatives docking on CYP1A1 was determined using EAdock[29] and Swissdock.[30] The analysis of the obtained binding modes was through UCSF Chimera.[31]

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