

## Synthesis, crystal structure, molecular docking and cytotoxicity of Zwitterionic 3-(4-amino-3-imino-5-oxo-2,3,4,5-tetrahydro-[1,2,4]triazin-6-yl)-propionic acid

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A new 3-(4-amino-3-imino-5-oxo-2,3,4,5-tetrahydro-[1,2,4]triazin-6-yl)-propionic acid monohydrate has been synthesised by the condensation reaction of  $\alpha$ -ketoglutaric acid with diaminoguanidine. The compound exists as zwitter ion, with the hydrogen atom of carboxylic group being transferred to the exocyclic imine nitrogen atom of triazine moiety. The zwitterionic condensed product was characterised by single crystal X-ray diffraction, elemental analysis, FT-IR, UV-Vis NMR and Mass spectroscopies, and TG-DTA methods. This salt crystallises in monoclinic system of space group  $P2_1/c$  with  $a = 6.4871(3)$  Å,  $b = 9.1562(5)$  Å,  $c = 14.9555(8)$  Å,  $\beta = 97.519(2)^\circ$ ,  $Z = 4$  and  $R1/wR2 [I \geq 2\sigma(I)] = 0.0512/0.1092$ . The structural units are held together by extensive array of N-H...O, O-H...O and C-H...O interactions and lattice water acts as a linker. The epidermal growth factor receptor (EGFR) is a tyrosine kinase receptor that is frequently expressed in epithelial tumours. The EGFR was the first receptor to be proposed as a target for cancer therapy, and the compound was subjected into the target protein in molecular docking approach. It was observed that the compound decreased the viability of MCF-7 cells in dose dependent level with the  $IC_{50}$  value of 15.2  $\mu\text{g/mL}$  and the morphology studies indicate that the compound induces apoptosis.

Keywords: Zwitterion, supramolecular network, EGFR tyrosine kinase, cytotoxic activity.

### Introduction

The 1,2,4-triazines are nitrogen-containing heterocycles possessing considerable interest because of high biological activities such as pesticide properties (herbicides, fungicides, insecticides, plant growth stimulants and inhibitors) and pharmaceutical activities, e.g. neurotropic, cardiotropic, bronchodilatory, vasodilatory, antifungal and anthelmintic ones<sup>1,2</sup>. Analysis of literature shows that 3-substituted 1,2,4-triazine-5 ones are mainly synthesized by cyclocondensation of  $\alpha$ -keto acids with thiosemicarbazides and thiocarbonylhydrazide and hydrazine derivatives<sup>3-5</sup>. The reactions are performed in two stages: initial formation of the corresponding  $\alpha$ -keto acid hydrazones followed by their cyclization to give the target triazines. The synthetic potential of this method is limited because the starting  $\alpha$ -keto acids obtained by multi-stage procedures are hardly available<sup>3-5</sup>. Bis-hydrazones derived from carbon- and thiocarbonohydrazides having both open chain and closed macrocyclic systems were studied<sup>6,7</sup>.

Our research group has been focusing on the condensation reaction and salt forming ability of hydrazine derivatives with variety of carboxylic acids<sup>8-11</sup> and carbonyl compounds<sup>12</sup>. Our recent report showed that the reaction between aminoguanidine and oxalic/sulpho acetic acid has led to zwitterionic salt via condensation<sup>13</sup>. In this line, we are interested in finding both condensation and neutralisation ability of hydrazine derivatives by careful selection of starting compounds.  $\alpha$ -Ketoacids are of wonderful candidates and possess interesting biological properties. Similarly, diaminoguanidine (DAG), diacidic base is known to have extensive applications in both chemical and biological systems<sup>14</sup>. Lieter and Strojny<sup>15</sup> synthesised triazine by condensing  $\alpha$ -dicarbonyl compounds, in particular benzil with diaminoguanidine with very low yield. To the best of our knowledge no attempt has been made to synthesise molecular salt based on DAG, except the pyruvate Schiff base derived from aminoguanidine that has been reported in its zwitterionic form by Turta *et al.*<sup>16</sup>. The aim of the present work is to

investigate the structural versatility of Schiff base to form supra molecular hydrogen bonding assemblies. And we report here the synthesis, crystal structure, molecular docking and cytotoxicity of zwitterionic triazine derivative.

## Experimental

### *Material and methods:*

All reagents and chemicals used were of analytical reagent grade (A.R.) and of highest purity. Doubly distilled water was used as a solvent for preparation. Elemental analysis for C, H, and N were performed on a Vario-ELIII elemental analyser. The IR spectrum was recorded on a JASCO-4100 spectrophotometer as KBr pellets in the range of 4000–400  $\text{cm}^{-1}$ . NMR spectra were recorded on a Bruker Avance III spectrometer operating at 400 MHz for  $^1\text{H}$  and 100 MHz for  $^{13}\text{C}$ .  $^1\text{H}/^{13}\text{C}$  NMR chemical shifts are reported in ppm and tetramethyl silane (TMS) used as an internal reference. Mass spectrum was performed on high resolution JEOL GCMATE II GC-MS double focusing instrument with electron impact ionisation mass spectrometer. Simultaneous TG-DTA studies were done on a Perkin-Elmer Pyris Diamond thermal analyser and the curves obtained in air using platinum cups as holders with 3 mg of the sample at the heating rate of  $10^\circ\text{C}/\text{min}$ . Absorption spectral analysis were performed using JASCO V-630 UV-Visible spectrophotometer with quartz cuvettes of path length 1 cm.

The single crystal used for the structure determination of the compound was found to be a non-merohedral twin with two components. The integration using Apex2, resulted in a total of 37181 reflections. 6486 reflections (853 unique) involved component 1 only (mean  $I/\sigma = 16.9$ ), 6520 reflections (860 unique) involved component 2 only (mean  $I/\sigma = 13.4$ ), and 24175 reflections (2301 unique) involved both components (mean  $I/\sigma = 14.9$ ). The data were corrected for absorption using Twinabs<sup>17</sup>. Using Olex2<sup>18</sup>, the structure was solved by direct methods (SHELXT)<sup>19</sup> with only the non-overlapping reflections of component 1. The structure was refined with the ShelXL<sup>20</sup>, using the HKLF 5 routine resulting in a BASF value of 0.3974(18). All hydrogen atoms including hydrogen atoms of water molecules are located with the riding model.

### *Preparation:*

An aqueous solution (20 mL) of 1,3-diaminoguanidine

monohydrochloride (0.125 g, 1 mmol) was added to 20 mL aqueous solution containing  $\alpha$ -ketoglutaric acid (0.146 g, 1 mmol) and stirred for an hour. The resulting clear solution was concentrated over a waterbath to reduce half of its volume and then kept at room temperature for crystallisation. Light pink colour crystals formed after three days were separated, washed with cold methanol and air dried. Yield: 89%, elemental analysis (Found: C, 33.65; H, 5.67; N, 32.55.  $\text{C}_6\text{H}_{11}\text{N}_5\text{O}_4$  Calcd.: C, 33.18; H, 5.10; N, 32.24%); UV-Vis ( $10^{-3}\text{M}$ ,  $\lambda_{\text{max}}$ , nm ( $\epsilon$  [ $\text{M}^{-1}\text{cm}^{-1}$ ])): 243 (23200), 303 (20800); FT-IR (KBr,  $\text{cm}^{-1}$ ): 3420 (OH), 3250 (NH), 1661 (C=N), 1613, 1422 ( $\text{COO}^-$ ), 1050 (N-N);  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ ,  $\delta$ , ppm): 7.07(s, NH), 5.50 (s,  $\text{H}_2\text{O}$ ), 2.79–2.76 (t, 7.0 Hz,  $\text{CH}_2$ ), 2.58–2.54 (t, 7.0 Hz,  $\text{CH}_2$ );  $^{13}\text{C}$  NMR (100 MHz,  $\text{DMSO}-d_6$ ): 163.19 (C8), 152.88 (C4), 140.17 (C5), 134.06 (C1), 20.19 (C7), 20.0 (C6).

### *Molecular docking study:*

The chemically synthesized compound is docked against the target protein tyrosine kinase (PDB: 1M17). The binding of small molecule to large protein targets is central to numerous biological processes and the accurate prediction of the binding modes between the ligand and protein is of fundamental importance in modern structure based drug design. Python 2.4-language was downloaded from <https://www.python.org/download/releases/2.4/>, Cygwin (data storage) was simultaneously downloaded from [www.cygwin.com](http://www.cygwin.com), Molecular graphics laboratory (MGL) tools and Auto Dock 1.5.6 was downloaded from <http://mgltools.scripps.edu/Support>, Chem sketch was downloaded from <http://www.acdlabs.com/resources/freeware/chemsketch/>.

### *Evaluation of cytotoxicity:*

The inhibitory concentration ( $\text{IC}_{50}$ ) value was evaluated using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. The medium was replaced with fresh medium containing serially diluted synthesized compound, and the cells were further incubated for 48 h. The culture medium was removed, and 100  $\mu\text{L}$  of the MTT solution was added to each well and incubated at  $37^\circ\text{C}$  for 4 h. After removal of the supernatant, 50  $\mu\text{L}$  of DMSO was added to each of the wells and incubated for 10 min to solubilize the formazan crystals. The optical density was measured at 620 nm in an ELISA multi well plate reader (Thermo Multiskan EX, USA). The OD value was used to calculate the percent-

age of viability using the following formula.

$$\% \text{ of viability} = \frac{\text{OD value of experimental sample}}{\text{OD value of experimental control}} \times 100 \quad (1)$$

#### Induction of apoptosis:

The MCF-7 cells that were grown on cover slips ( $1 \times 10^5$  cells/cover slip) incubated for 6–24 h with compound at the  $IC_{50}$  concentration, and they were then fixed in an ethanol:acetic acid solution (3:1; v/v). The cover slips were gently mounted on glass slides for the morphometric analysis. Three monolayers per experimental group were photo micrographed. The morphological changes of the MCF-7 selected cells were analyzed using Nikon (Japan) bright field inverted light microscopy at 40x magnification. Approximately 1  $\mu$ L of a dye mixture (100 mg/mL acridine orange (AO) and 100 mg/mL ethidium bromide (EtBr) in distilled water) was mixed with 9 mL of cell suspension ( $1 \times 10^5$  cells/mL) on clean microscope cover slips. The selected cancer cells were collected, washed with phosphate buffered saline (PBS) (pH 7.2) and stained with 1 mL of AO/EtBr. After incubation for 2 min, the cells were washed twice with PBS (5 min each) and visualized under a fluorescence microscope (Nikon Eclipse, Inc, Japan) at 400x magnification with an excitation filter at 480 nm. Likewise the cells were placed on glass coverslip in a 24-well plate and treated with compound for 24 h. The fixed cells were permeabilised with 0.2% Triton X-100 (50  $\mu$ L) for 10 min at room temperature and incubated for 3 min with 10  $\mu$ L of DAPI by placing a coverslip over the cells to enable uniform spreading of the stain. The cells were observed under (Nikon Eclipse, Inc, Japan) fluorescent microscope.

#### Results and discussion

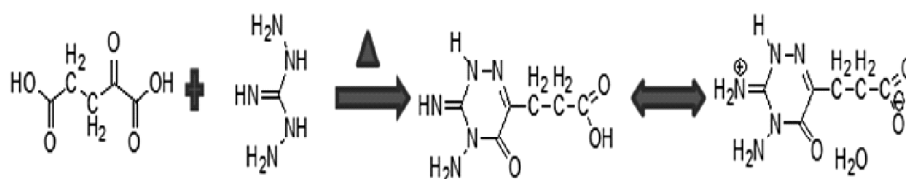
The triazine derivative was prepared by the direct condensation of  $\alpha$ -ketoglutaric acid with diaminoguanidine in

aqueous medium. In this reaction both condensation and neutralisation are possible. Initially condensation prevails over neutralisation resulting Schiff base formation and in turn internal condensation (ring closure reaction) occur leading to triazine compound. Subsequently, there is a proton transfer from the carboxyl group to the exocyclic imine part of triazine. The synthetic route of new zwitterionic triazine is outlined in Scheme 1. The compound is stable in air and highly soluble in ethanol, methanol and water. Our attempt to prepare this compound in methanol, ethanol and acetonitrile was unsuccessful. Also the reaction of aminoguanidine with  $\alpha$ -ketoglutaric acid did not yield any desired product.

#### Structural description:

The molecular structure with atom labelling scheme of the salt is depicted in Fig. 1. The compound crystallizes in monoclinic system in the space group  $P2_1/c$  with  $Z = 4$ . Crystallographic data are presented in Table 1 and selected bond parameters are summarized in Table S1 and S2. The asymmetric unit consists of zwitterionic condensed salt and a water molecule. The C5-N1 bond distance is 1.289(3) Å that is appreciably close to that of C=N confirming the formation of Schiff base<sup>16,21</sup>. The triazine ring and the carboxylate group are arranged in same plane in the structure. In addition, a proton transfer occurs from propionic part of ketoglutaric acid to imine nitrogen of guanidine moiety resulting internal salt formation. This was confirmed by the variation in the C-O bond distances  $d_{C-O}$  of the carboxylate group i.e. 1.246(3) (C8-O9), 1.282(3) (C8-O8) Å in relation to its asymmetric nature<sup>13</sup>.

Similarly three crystallographically independent C-N bond lengths in the guanidinium moiety (1.312(3) (C1-N3), 1.342(3) (C1-N2), 1.362(3) (C1-N4) Å) of triazine was observed. The results are comparable to that of, e.g. diaminoguanidinium sulphate<sup>22</sup>, diamino-guanidiniumazotetrazolate<sup>23</sup> and diaminoguanidinium 3-nitro-1,2,4-triazol-5-one<sup>24</sup>.



**Scheme 1.** Synthetic route of triazine derivative.

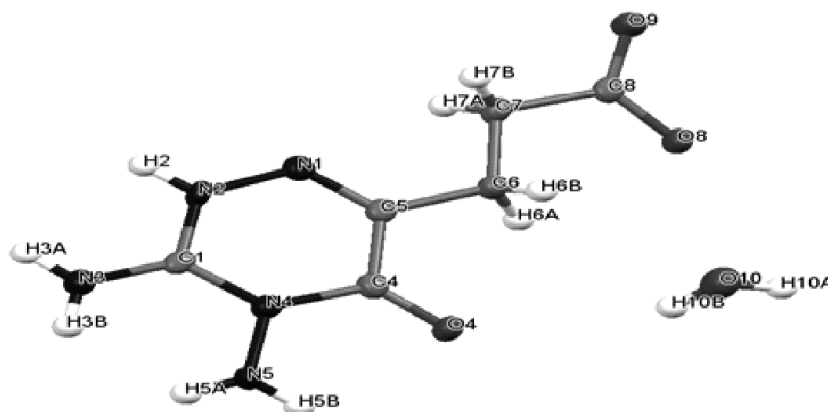


Fig. 1. Molecular structure of the compound.

Table 1. Crystallographic data

Compound	
Empirical formula	C <sub>6</sub> H <sub>11</sub> N <sub>5</sub> O <sub>4</sub>
Formula weight	217.20
Temperature (K)	100.0
Crystal system	monoclinic
Space group	P2 <sub>1</sub> /c
<i>a</i> (Å)	6.4871(3)
<i>b</i> (Å)	9.1562(5)
<i>c</i> (Å)	14.9555(8)
$\alpha$ (°)	90
$\beta$ (°)	97.519(2)
$\gamma$ (°)	90
Volume (Å <sup>3</sup> )	880.68(8)
<i>Z</i>	4
$\rho_{\text{calc}}$ (g/cm <sup>3</sup> )	1.638
$\mu$ (mm <sup>-1</sup> )	0.138
<i>F</i> (000)	456.0
Crystal size (mm <sup>3</sup> )	0.25×0.2×0.15
Radiation	MoK $\alpha$ ( $\lambda$ = 0.71076)
2 $\theta$ range for data collection (°)	6.336 to 56.624
Index ranges	$-8 \leq h \leq 8, 0 \leq k \leq 12, 0 \leq l \leq 19$
Reflections collected	2249
Independent reflections	2249 [ $R_{\text{int}}$ = 0.0507, $R_{\text{sigma}}$ = 0.0303]
Data/restraints/parameters	2249/0/141
Goodness-of-fit on $F^2$	1.121
Final <i>R</i> indexes [ $I > 2\sigma(I)$ ]	$R_1$ = 0.0512, $wR_2$ = 0.1092
Final <i>R</i> indexes [all data]	$R_1$ = 0.0642, $wR_2$ = 0.1161
Largest diff. peak/hole/e Å <sup>-3</sup>	0.47/−0.50

In the crystal, symmetry related molecules are hydrogen bonded via carboxylate oxygen (O9) and the amino hydrogen (N5-H5B). This results in the formation of one dimensional chain running parallel to *b*-axis when viewed down-*a* (Fig. 2). Lattice water plays a vital role in strengthening the network, in which it acts as a bifurcated acceptor to the amino group. In this chain, each molecule is linked by N-H...O hydrogen bonds through N3-H3B...O10 and N5-H5A...O10 to generate  $R_2^1(7)$  ring motif as shown in Fig. 2.

Furthermore, the anionic and cationic parts of the zwitter ion are hydrogen bonded so as to create  $R_1^2(4)$ ,  $R_2^1(6)$ ,  $R_2^2(8)$  ring motifs achieved through N-H...O interactions, thus creating 2D layer structure. The antiparallel sheets are connected by C6-H6B...O9 (3.48 Å) interactions resulting in three dimensional supramolecular network as shown in Fig. 3.

#### Spectral characterization:

The <sup>1</sup>H and <sup>13</sup>C NMR spectra have been recorded for the compound in DMSO-*d*<sub>6</sub> solution and are shown in Figs. 4 and 5. In <sup>1</sup>H NMR spectrum, appearance of a broad signal at 7.07 ppm has been assigned to NH protons. A peak at 5.50 ppm with an integration corresponds to two protons is assigned for a lattice water molecule. The methylene protons of ligand nearer to carboxylate and azomethine groups are observed in the region between 2.79–2.76 and 2.58–2.54 ppm respectively. The <sup>13</sup>C NMR spectrum revealed six signals that are due to the six magnetically different carbons present in the compound. The carboxylate (COO<sup>−</sup>) and carbonyl (C=O) carbon signals appeared in the most downfield shift at 163 and 152 ppm respectively. Two signals at 140

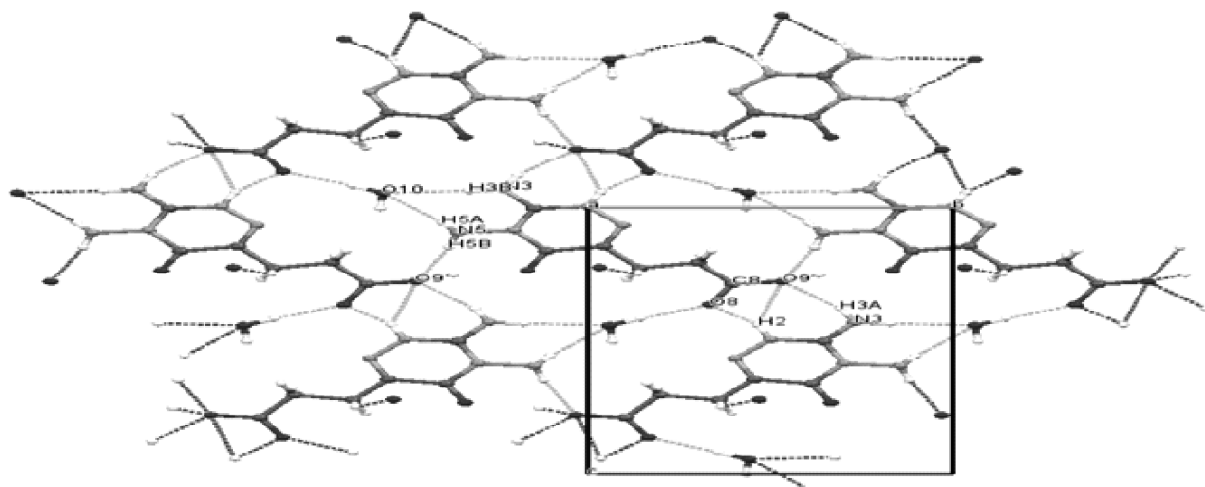


Fig. 2. Crystal packing of compound viewed down a-axis.

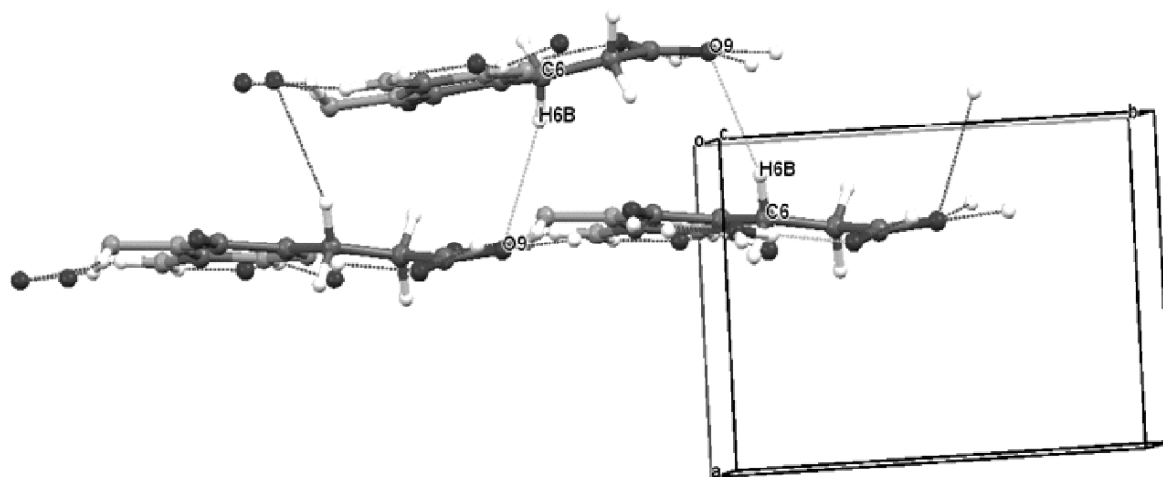


Fig. 3. Third dimensional created by C-H...O interactions - down b.

and 134 ppm corresponds to azomethine carbons of triazine. A doublet at 20 ppm is assigned to methylene carbons.

Mass spectrometry is a micro analytical technique that provides characteristic information pertaining to the structure and molecular weight of the compounds. Mass spectrum of the compound (Fig. 6) shows peak at 217.4 matching exactly with the composition of the compound. A base peak at  $m/z$  199 corresponds to the anhydrous zwitterion which again confirm the overwhelming stability.

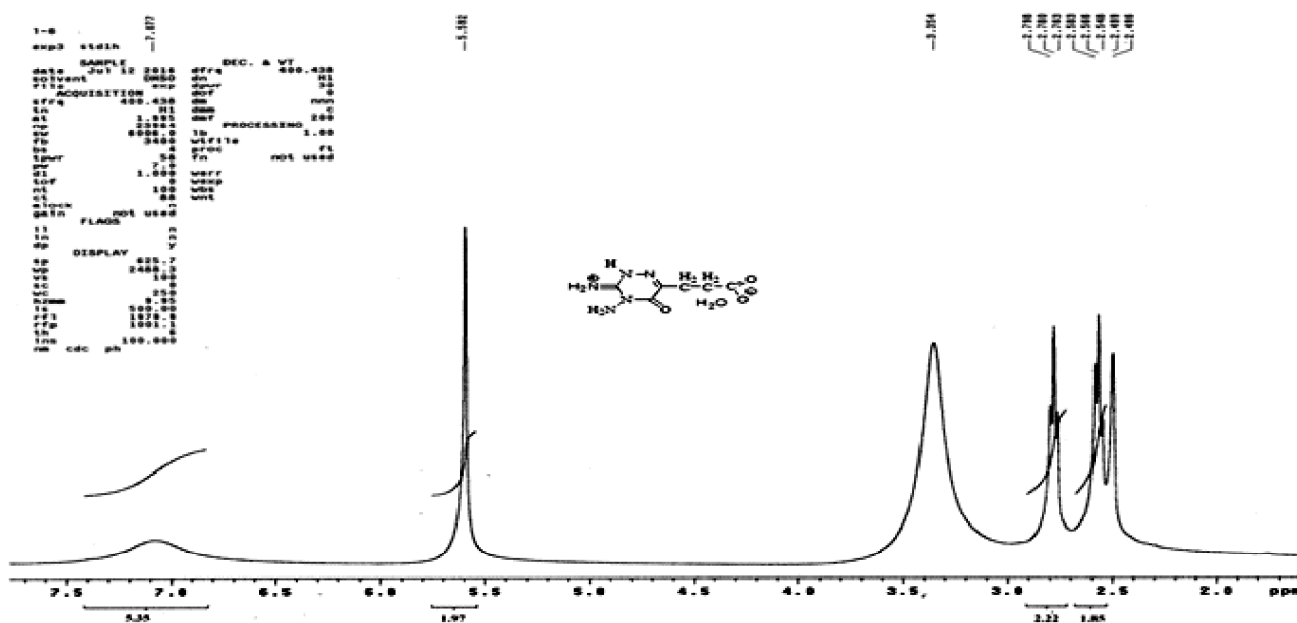
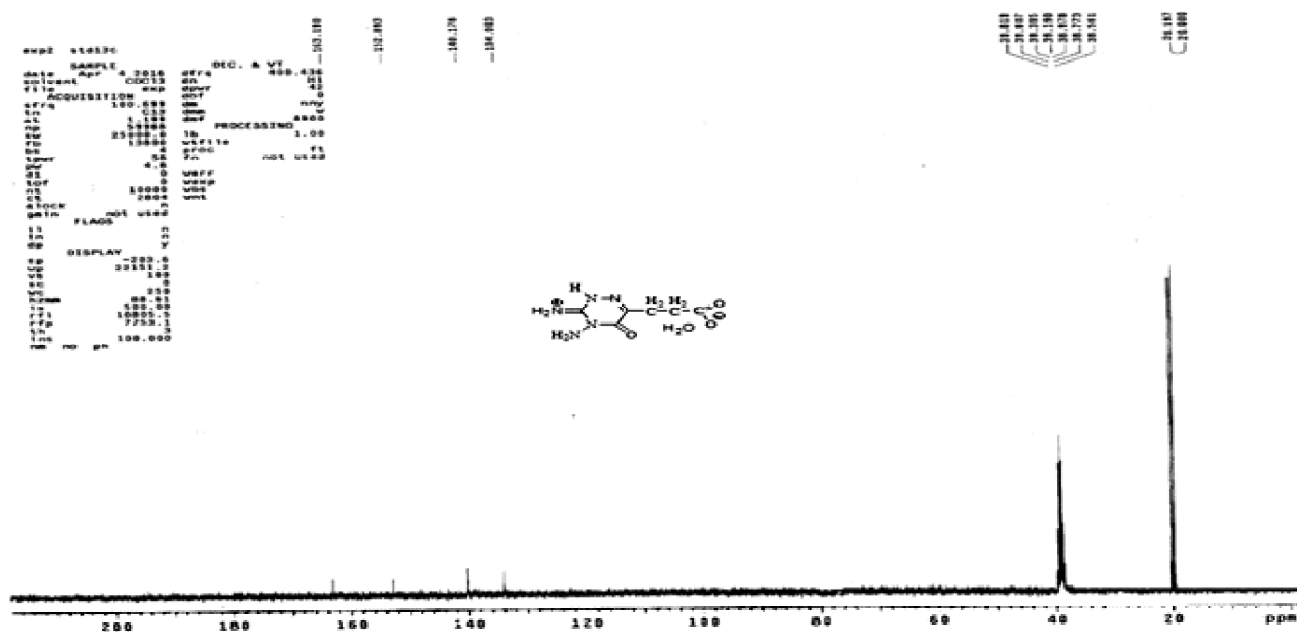
#### Thermal study:

Thermal analysis provides information regarding the thermal stability of the compound. The TG exhibits three distinct steps of decomposition in accordance with DTA (Fig. S1).

Initially, the compound shows an endotherm in DTA at 92°C corresponding to dehydration of lattice water molecule with the weight loss of 8.00% (Calcd. 8.33%). The anhydrous compound undergoes decarboxylation endothermically (265°C) resulting triazine derivative ( $C_5H_9N_4O$ : Obsd. 28.00%; Calcd. 28.57%) as an intermediate, which further decomposes exothermically at 615°C to give gaseous end products.

#### Molecular docking studies:

The epidermal growth factor receptor (EGFR) defines a family of tyrosine kinase receptors (TKRs) including ErbB2/HER2, ErbB3/HER3 and ErbB4/HER4<sup>25,26</sup>. As a cell surface protein that binds to epidermal growth factor, its binding to a ligand induces receptor dimerization and tyrosine auto phos-

Fig. 4.  $^1\text{H}$  NMR spectrum in  $\text{DMSO}-d_6$  solvent.Fig. 5.  $^{13}\text{C}$  NMR spectrum in  $\text{DMSO}-d_6$  solvent.

phorylation and leads to cell proliferation, of which altered activity has been implicated in the development and growth of many tumours<sup>27</sup>. EGFR is the cell-surface receptor and cancer biomarkers. Its over expression or over activity has been associated with a number of cancers, including breast, lung, ovarian, and anal cancers. Many therapeutic ap-

proaches are aimed at the EGFR<sup>28</sup>. A variety of evidence suggests that over expression of EGFR has been associated with oncogenic activity such as breast, colorectal, ovarian and non-small cell lung cancers<sup>29</sup>. Since then EGFR has been identified as a promising target for the treatment of many human cancers. The development of potent EGFR

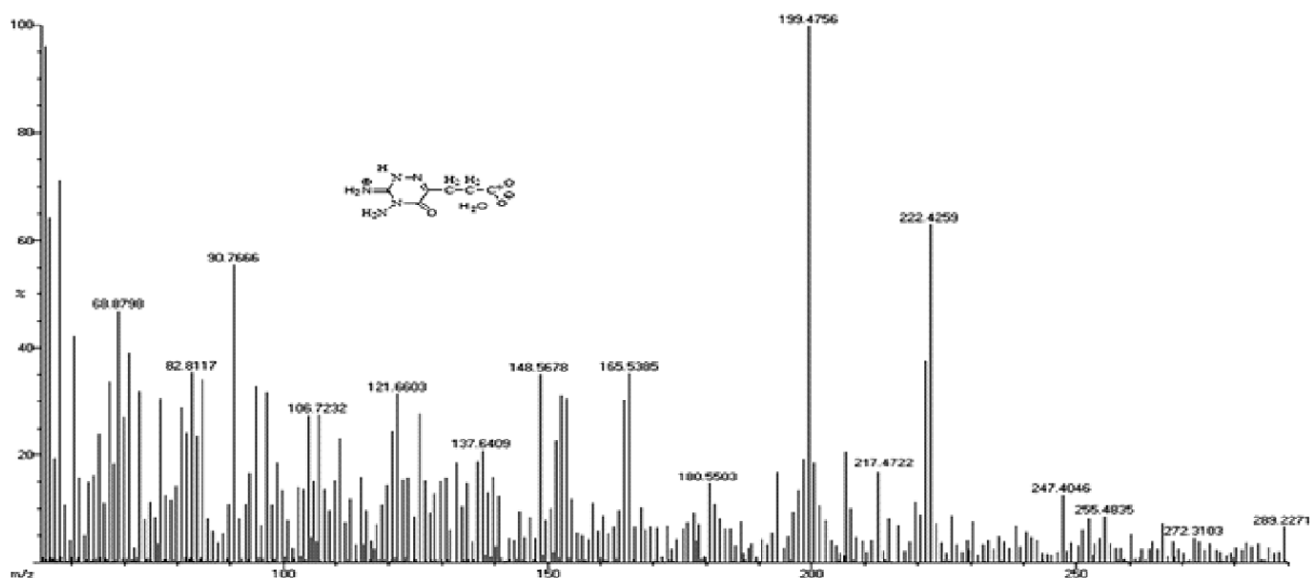


Fig. 6. Mass spectrum of the compound.

inhibitors has become an increasingly attractive area for discovering new anti-cancer drugs. Recently, different compounds have been investigated as novel EGFR inhibitors<sup>30–34</sup>, which include thienopyrimidine, thiazolo[4,5-*d*]pyrimidine, arylaminopyrimidine, pyrazolo[3,4-*d*]pyrimidine, and pyrrolotriazine derivatives. Therefore, studies on the TKIs-EGFR interactions might provide some useful insights or clues for developing effective anti-EGFR drugs.

To gain more understanding on structure-activity relationships observed for EGFR-tyrosine kinase, molecular docking was performed on binding model of EGFR (PDB: 1M17). Analysis of docking results revealed that the compound possesses binding energy of  $-10.12$  kcal/mol. The 2D binding model of the compound with EGFR receptor is depicted in Fig. 7. In the binding model, compound bound through amino acid residues resulting two hydrogen bonds. Hydrogen bond interactions between receptor and compound are shown as dotted lines in Fig. 7. One conventional hydrogen bond was observed between the nitrogen atom of triazine ring moiety with the oxygen atom of Asp 950 and another hydrogen bond was formed between the carboxylate oxygen and nitrogen atom of Arg 953. This attributed to the reason that compound showed good inhibition against EGFR kinase receptor. Compound is well interacted with cancer cells through EGFR receptors rather than the normal cells. These findings have provided a rationale for the development of novel anticancer agents that target EGFR receptor.

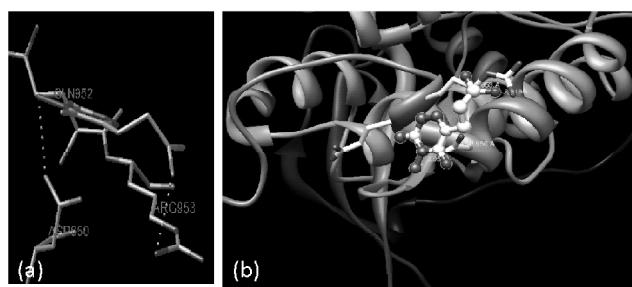


Fig. 7. Docked conformation of EGFR with compound (a); binding modes of compound (b).

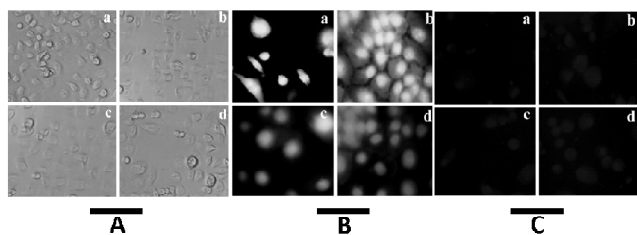
#### Cytotoxicity:

Historically, the biomedical properties of triazine compounds were previously investigated as anticancer drug<sup>3–5</sup>. Therefore the salt was screened for cytotoxicity against MCF-7 by MTT method using Doxorubicin as a reference. It displayed potent activity with the  $IC_{50}$  value of  $15.2 \mu\text{g/mL}$ . It was further examined for possible cytotoxicity against HBL-100 (normal breast cell line) and found that the compound exhibited weak cytotoxicity against HBL-100 ( $21.23 \mu\text{g/mL}$ ). The results indicated that compound had good selectivity between the selected cancer cell line (MCF-7) and a normal cell line (HBL-100).

#### Induction of apoptosis:

To induce apoptosis, MCF-7 human breast cancer cells were cultured in the presence of increased concentration of

triazine compound and cellular morphology was observed with a phase contrast microscope (Fig. 8A). While the control cells have a round morphology, phase-contrast micrographs reveal that the compound induces increased cell shrinkage, membrane blebbing and forms floating cells, compared to the control in a dose-dependent manner (Fig. 8A(b to d)). Chromatin condensation was also detected using the fluorescent DNA binding dye (AO/EtBr). As shown in Fig. 8B, the untreated MCF-7 cancer cells (control) did not show any significant adverse effect compared to the compounds treated cancer cells (Fig. 8B(b-d)). It can be observed that with the addition of compounds (b-d) to the MCF-7 cancer cells, the green colour of cells are converted into orange/red which is due to induced apoptosis and the nuclear condensation effect. Further confirmation of apoptosis came from the DAPI staining assay (Fig. 8C). Compound treated MCF-7 cancer cells show bright fetches which indicate the condensed chromatin and nuclear fragmentations and it is comparable to the control.



**Fig. 8.** Morphological study and DNA fragmentation of K562 cells treated with 6 mg/mL of the compound for 72 h. (A) Phase contrast images of the MCF-7 cells. The control cells have completely crowded and attached form, while the treated cells obviously crowded decreased and unattached, condensed and fragmented. (B) Fluorescent microscopy of AO/EtBr double staining apoptosis assay. Green colours of cells are converted into orange/red colour cells which are due to induced apoptosis. (C) DAPI staining assay, cancer cells shows bright fetches which indicates the condensed chromatin and nuclear fragmentations in the cancer cells.

## Conclusion

The presence of both amino and imino groups in diaminoguanidine affords both the protonation and condensation. Single crystals of zwitterionic triazine were obtained by adopting simple condensation reaction. The compound was characterised by elemental analysis, FT-IR, UV-Vis, NMR and Mass spectroscopies, and TG-DTA methods and structure was confirmed by X-ray diffraction technique. In air, it exhibited subsequent endo-followed by exothermic decom-

position to give gaseous products in the temperature range 100–650°C.

The supra molecular structure was stabilized by a variety of intermolecular contacts including N-H...O, O-H...O, C-H...O interactions that generate three-dimensional network. The carboxylate oxygen (O9) acts as tetrafurcated acceptor and favours the creation of 3D network. Molecular docking studies with EGFR receptor revealed good binding affinity with an energy of –10.12 kcal/mol and resulting two conventional hydrogen bonds towards preferential amino acid residues. The compound displayed potent activity with the IC<sub>50</sub> value of 15.2 µg/mL and morphology studies further confirmed the induced apoptosis.

## Acknowledgement

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## Supplementary data

The following is the supplementary data related to this article: CCDC 1563443 contains the supplementary crystallographic data for compound. These data can be obtained free of charge via <http://www.ccdc.cam.ac.uk/conts/retrieving.html>, or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; Fax: (+44) 1223-336-033; or E-mail: [deposit@ccdc.cam.ac.uk](mailto:deposit@ccdc.cam.ac.uk).

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