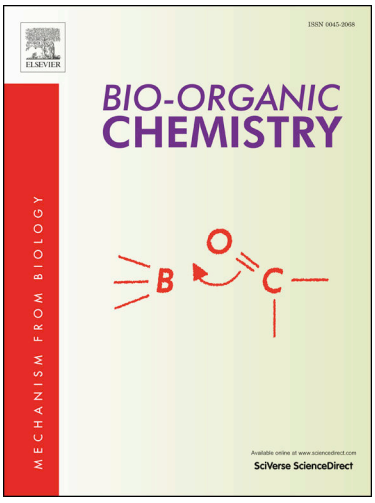
Journal Pre-proofs



Di- and tri-substituted *s*-triazine derivatives: Synthesis, characterization, anti-cancer activity in human breast-cancer cell lines and developmental toxicity in zebrafish embryos

Ayman El-Faham, Muhammad Farooq, Zainab Almarhoon, Rakia Abd Alhameed, Mohammad A.M. Wadaan, Beatriz G. de la Torre, Fernando Albericio

PII: S0045-2068(19)31245-3
DOI: https://doi.org/10.1016/j.bioorg.2019.103397
Reference: YBIOO 103397

To appear in: *Bioorganic Chemistry*

Received Date: 31 July 2019
Revised Date: 15 September 2019
Accepted Date: 22 October 2019

Please cite this article as: A. El-Faham, M. Farooq, Z. Almarhoon, R. Abd Alhameed, M.A.M. Wadaan, B.G. de la Torre, F. Albericio, Di- and tri-substituted *s*-triazine derivatives: Synthesis, characterization, anticancer activity in human breast-cancer cell lines and developmental toxicity in zebrafish embryos, *Bioorganic Chemistry* (2019), doi: https://doi.org/10.1016/j.bioorg.2019.103397

Di- and tri-substituted *s*-triazine derivatives: Synthesis, characterization, anticancer activity in human breast-cancer cell lines and developmental toxicity in zebrafish embryos

Ayman El-Faham,[a,b,*] Muhammad Farooq, [c] Zainab Almarhoon,[a] Rakia Abd Alhameed,[a] Mohammad A.M. Wadaan,[c] Beatriz G. de la Torre,[d] Fernando Albericio[a,e,f,*]

[a]*Department of Chemistry, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia*
[b]*Department of Chemistry, Faculty of Science, Alexandria University, P.O. Box 426, Alexandria 21321, Egypt*
[c]*Bioproducts Research Chair, College of Science, Department of Zoology, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia*
[d]*KRISP, College of Health Sciences, University of KwaZulu-Natal, Westville, Durban 4001, South Africa*
[e]*School of Chemistry and Physics, University of KwaZulu-Natal, University Road, Westville, Durban 4001, South Africa*
[f]*CIBER-BBN, Networking Centre on Bioengineering, Biomaterials and Nanomedicine, and Department of Organic Chemistry, University of Barcelona, Martí i Franqués 1-11, Barcelona 08028, Spain*

Abstract: Here we report on a small library based on a 4-aminobenzonitile-*s*-triazine moiety. We used a straightforward orthogonal synthetic pathway to prepare di- and tri-substituted *s*-triazine derivatives, whose basic structure was modified. The newly synthesized compounds were fully characterized by ^{1}H-NMR, ^{13}C-NMR and elemental analysis. They showed strong anticancer activity against two human breast cancer cell lines (MIDA-MB-231 and MCF-7), with IC_{50} values less than 1 μM. These s-triazine compounds were generally more selective towards hormone receptor-positive breast cancer cell line MCF-7 than the triple negative MDA-MB-231 cell line. Zebrafish embryos were used to test the developmental toxicity of the target compounds *in vivo*. The phenotype of embryos treated with the derivatives resembled that of those treated with estrogen disruptors. This observation strongly supports the notion that that these compounds induce their anticancer activity in human breast cancer cells via targeting the estrogen and progesterone receptors.

Keywords: 4-aminobenzonitile-s-triazine, di- trisubstituted *s*-triazine, anticancer activity, zebrafish embryos

1. Introduction

Human diseases pose a major challenge for the pharmaceutical industry. In this context, chemistry laboratories worldwide are channeling efforts not only into synthesizing new types of molecules but also into modifying existing drugs in order to enhance activity and decrease toxicity.

1,3,5-Triazine (*s*-triazine) has drawn significant attention in organic chemistry due to its ease of handling and low cost of the starting material (cyanuric chloride)[1-8]. The *s*-triazine derivatives reported in the literature have been synthesized using methods that take advantage of the differences in the reactivity of the three chlorines in cyanuric chloride under temperature control [9, 10].

The 2,4,6-Trisubstituted *s*-triazine scaffold has played a key role in the medicinal chemistry field. Indeed, a number of *s*-triazine-based compounds show valuable biological activities [11-15]. In this regard, *s*-triazine derivatives bearing a 4-minobenzonitrile moiety show enhanced antimicrobial activity [16] and improved antitubercular properties over other chemical moieties, in addition to exerting strong anticancer activity [17]. Recent studies have confirmed that several *s*-triazine derivatives with a morpholine, piperidine and/or piperazine ring have significant biologically activity against *M. tuberculosis* strain H37Rv [18]. In addition, our group has reported that several trisubstituted *s*-triazine compounds show promising biological activity as anticancer agents [19-21].

In the drug discovery phase, in addition to addressing the biological activity of a target compound, attention should also be given to its potential toxicity. In this regard, the zebrafish has emerged as a valuable model in which to evaluate the potential toxicity and biological activity of new compounds [22]. The experiments conducted in early-stage zebrafish embryos (up to 120 hours post fertilization) do not require the approval of Institutional Animal Care and Use Committees (IACUCs) [23]. Therefore, a large number of zebrafish embryos can be used for in-depth statistical analysis, which would otherwise not be possible in other animal models due to restrictions on the numbers used for experimental purposes.

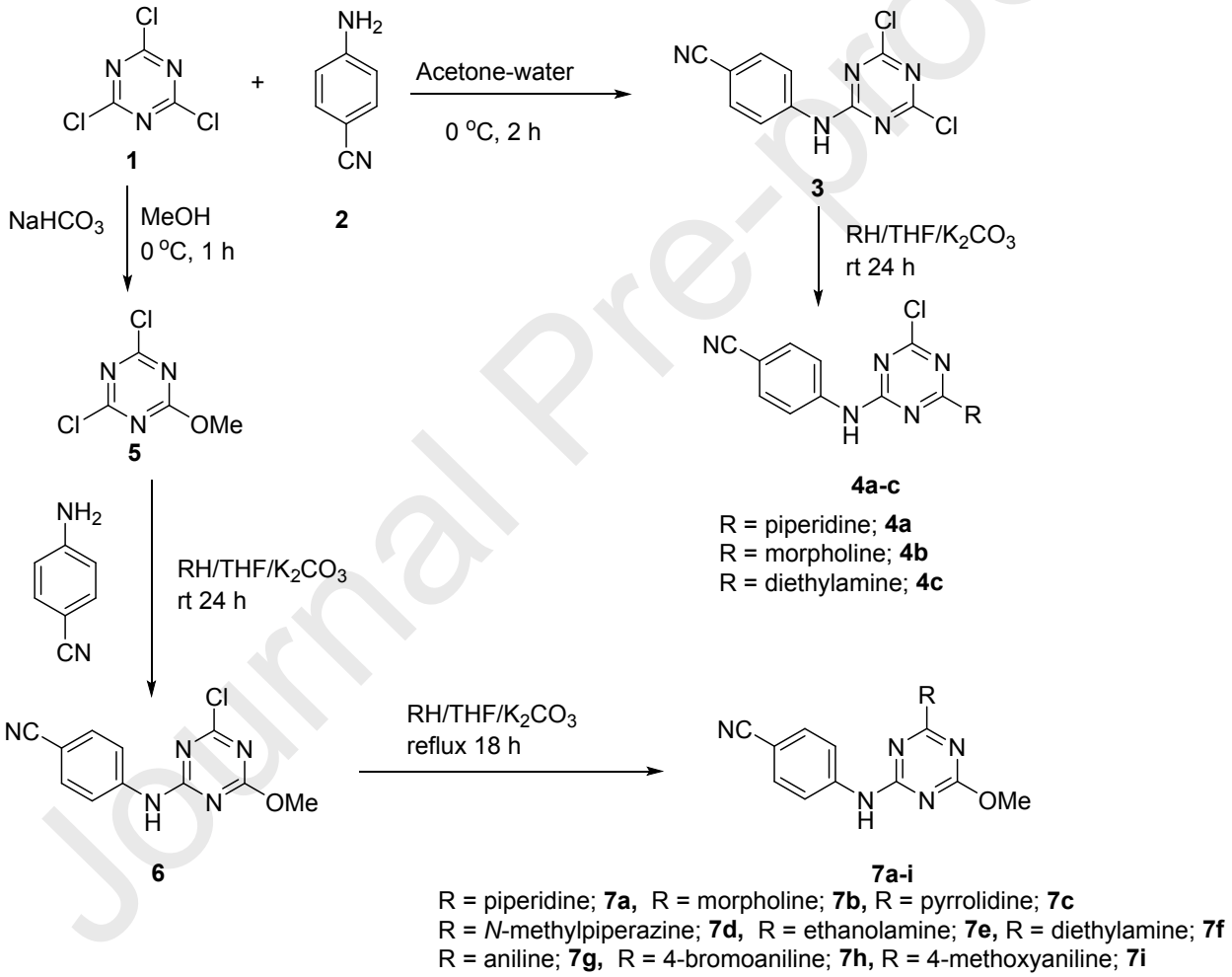
The potential positive effects of *s*-triazine and the imperative need to identify new chemical entities to feed drug discovery programs led us to use a straightforward orthogonal synthetic pathway to synthesize a small library based on 4-aminobenzonitrile *s*-triazine. This synthetic approach was used to prepare di- and tri-substituted *s*-triazine derivatives The newly synthesized compounds were then tested in two types of human breast cancer cell lines, namely MIDA-MB-

231 and MCF-7. Furthermore, their developmental toxicity *in vivo* was also evaluated using zebrafish embryos.

2. Results and discussion

2.1. Chemistry

In the first step of the the synthesis, cyanuric chloride (TCT, **1**) was reacted with 4-aminobenzonitrile **2** at 0°C for 2 h using acetone-water medium to afford product **3** in excellent yield and purity. Next, **3** was reacted with various amines using THF as solvent and K_2CO_3 as HCl scavengers to afford product **4a-c** in high yield and purity, as determined from their spectral data (Scheme 1).



Scheme 1. Synthesis of di-and tri-substituted 1,3,5-triazine derivatives

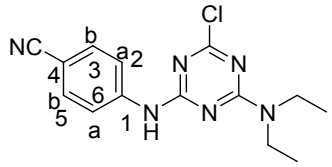


Fig. 1. Structure of **4c**

The ^{1}H NMR spectrum for compound **4c** (Figure 1, *Supporting information Figure S3*) as a prototype showed two multiple peaks at δ 1.09–1.18 and 3.51–3.59 ppm, related to $2CH_3$ and $2CH_2$ for the ethyl residue, respectively, a doublet at δ 7.74 ppm, related to two aromatic protons (*2Ha*), a doublet at δ 7.88 ppm, related to the other two aromatic protons ($2H_b$), and a broad singlet at δ10.46 ppm, corresponding to the NH. The ^{13}C NMR spectrum for **4c** showed four peaks at δ 12.8, 12.9, 41.5,41.9, related to the diethylamino group (CH_3, and CH_2 , respectively) and absorption peaks at δ 104.1(C_4), 119.2 (CN), 119.7($C_{2,6}$), 133.1 ($C_{3,5}$), 143.5(C_1), 163.3 (C-Cl), 168.3 (C-NH), and 175.5 (C-N) ppm.

In case of the methoxy derivatives **6** and **7a-i**, and to assure a high yield and purity from product **6**, TCT **1** was reacted with methanol in the presence of $NaHCO_3$ following the reported method [24], affording product **5**. Next, **5** was reacted with 4-aminobenzolnitrile **2** using the same conditions as described above to afford product **6** in high yield and purity. Compound **6** was then reacted with different amines in THF as solvent in the presence of K_2CO_3 as HCl scavengers under refluxing conditions for 18 h to afford product **7a-i** in high yield and purity. The spectral data and elemental analyses confirmed all the structures of the prepared compounds.

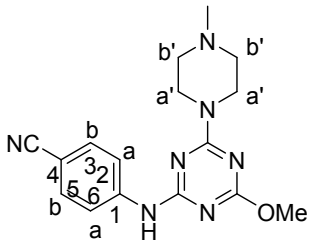


Fig. 2. **Structure of** 7d

The ^{1}H NMR for compound **7d** (Figure 2, *Supporting information, Figure S6*) as a prototype showed **a** singlet at δ 2.19 ppm (NCH_3), two broad singlets at δ 2.34 and 3.78 ppm, related the eight protons corresponding to $4CH_2$ ($H_{a'}$ and $H_{b'}$, respectively) piperazine residue, a singlet at δ

3.84 ppm (OCH_3), two doublets at δ 7.70 and 7.90 ppm, related to four aromatic protons (H_a, and H_b, respectively), and a singlet at δ 10.02 ppm, related to the NH. The ^{13}C NMR spectrum for **7d** showed the expected peaks at δ 42.8(N-CH_3), 45.7($C_{a'}$), 54.8 ($C_{b'}$), 54.2 (OCH_3), 103.3 (C-CN), 119.3 (CN), 119.4($C_{2,6}$), 132.9($C_{3,5}$), 144.2(C_1), 165.2 (C-Cl), 170.6 (C-NH), and 175.5 (C-N) ppm.

2.2. Biology

2.2.1. The triazine derivatives markedly *perturbed the proliferation of breast cancer cell lines*

s-Triazine-based compounds show promising anticancer activity [16,17, 25-27]. Here we performed some structural modifications in order to enhance this activity. Accordingly, the compounds synthesized here were based on an active substituent 4-aminbenzonitrile as first substituent, attached to *s*-triazine to generate two distinct series **4a-c** and **7a-i** (Scheme 1). To allow comparison of the activity of different nucleophiles, such as methoxy, acyclic amine, aliphatic and aromatic cyclic amine, in human breast cancer cells MCF-7 and MDA-MB-231, the type of substituent in the two series was selected on the basis of the reported structure-reactivity relationship [3,28,29] [30].

The anti-cancer activity of derivatives **4a-c** and **7a-7i** was tested in two human breast cancer cell lines: the MDA-MB-231 cell line, a triple negative breast cancer— meaning it does not express any kind of hormone receptors, and the MCF-7 cell line, which has estrogen and progesterone receptors.

The results of the MTT cell proliferation assay indicated that these compounds inhibited the proliferation of both cancer cell lines at minimum IC_{50} values. However, they showed much stronger activity in MCF-7 than MDA-MB- 231 cells (Table 1 and Figures 3-6). The most effective compounds were **7g** and **7i**, with IC_{50} values of 0.77 ± 0.01 and 0.1 ± 0.01 μM in the MCF-7 cell line and 8.43 ± 0.01 and 14.28 ± 0.01 μM in the MDA-MB-231 cell line, respectively. Moderate anticancer activity (IC_{50} values between 1 and 5 μM) was shown by **4c, 7b,** and **7f**, which had IC_{50} values of 1.3 ± 0.01, 4.291 ± 0.02, and 3.71 ± 0.04 μM in the MCF-7 cell line, and 25.60 ± 0.00, 7.80 ± 0.03, and 6.49 ± 0.04 μM, respectively in the MDA-MB-231 cell line. However, neither **7d** or **7e** showed activity in either cancer cell line.

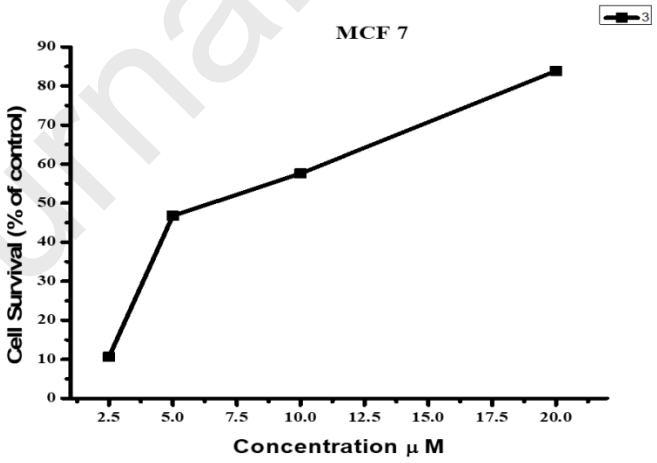
A similar type of anticancer activity and selectivity towards MCF-7 cells has also been described for *s*-triazine-bearing benzimidazole and benzothiazole derivatives. Those compounds,

showing IC_{50} values between 5–15 μM, impaired the proliferation of MCF7 cells [31]. In contrast, the triazine compounds reported herein, with IC_{50} values of less than μM, showed greater cytotoxicity towards MCF-7 cells.

Table 1. Cell proliferation assay of the triazine derivatives in two human breast cancer cell lines

Compd.	IC_{50} values (μM)* MDA-MB-231	MCF-7
3	23.11 ± 0.01	6.84 ± 0.03
6	19.08 ± 0.01	20.41 ± 0.0
4a	7.26 ± 0.03	10.12 ± 0.03
4b	10.61 ± 0.01	11.02 ± 0.03
4c	25.60 ± 0.00	1.3 ± 0.01
7a	21.30 ± 0.00	13.38 ± 0.02
7b	7.80 ± 0.03	4.29 ± 0.02
7c	10.79 ± 0.01	14.92 ± 0.02
7d	Not Active	Not Active
7e	Not Active	Not Active
7f	6.49 ± 0.04	3.71 ± 0.04
7g	8.43 ± 0.01	0.77 ± 0.01
7h	Not Active	9.44 ± 0.05
7i	14.28 ± 0.01	0.1 ± 0.01

***** These values are the mean of three replicates ± standard deviation.



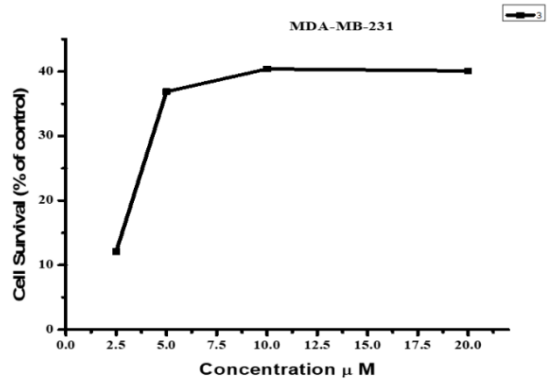


Fig. 3. Line graph showing the effect of compound **3** on the survival of MCF-7 and MDA-MB-231 human breast cancer cells

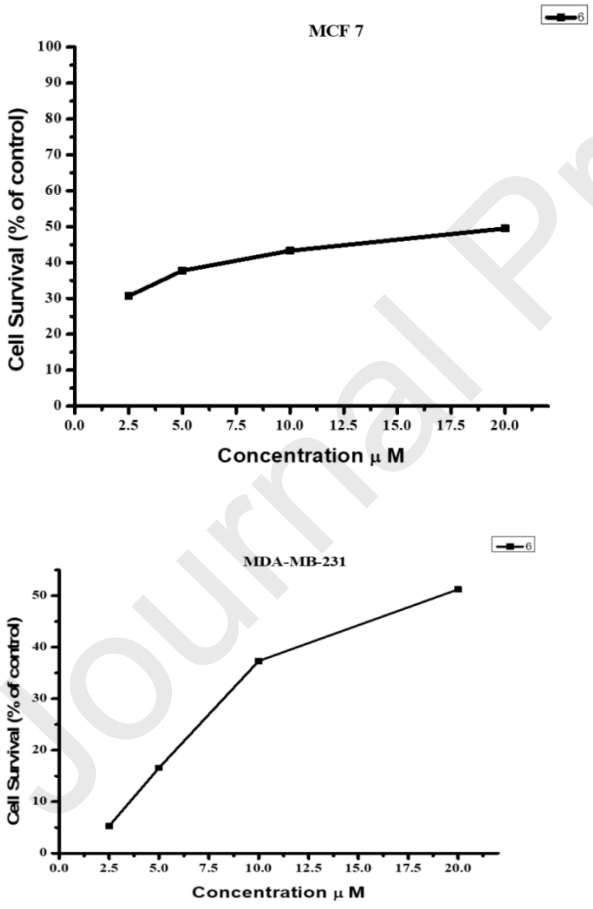


Fig. 4. Line graph showing the effect of compound **6** on the survival of MCF-7 and MDA-MB-231 human breast cancer cells

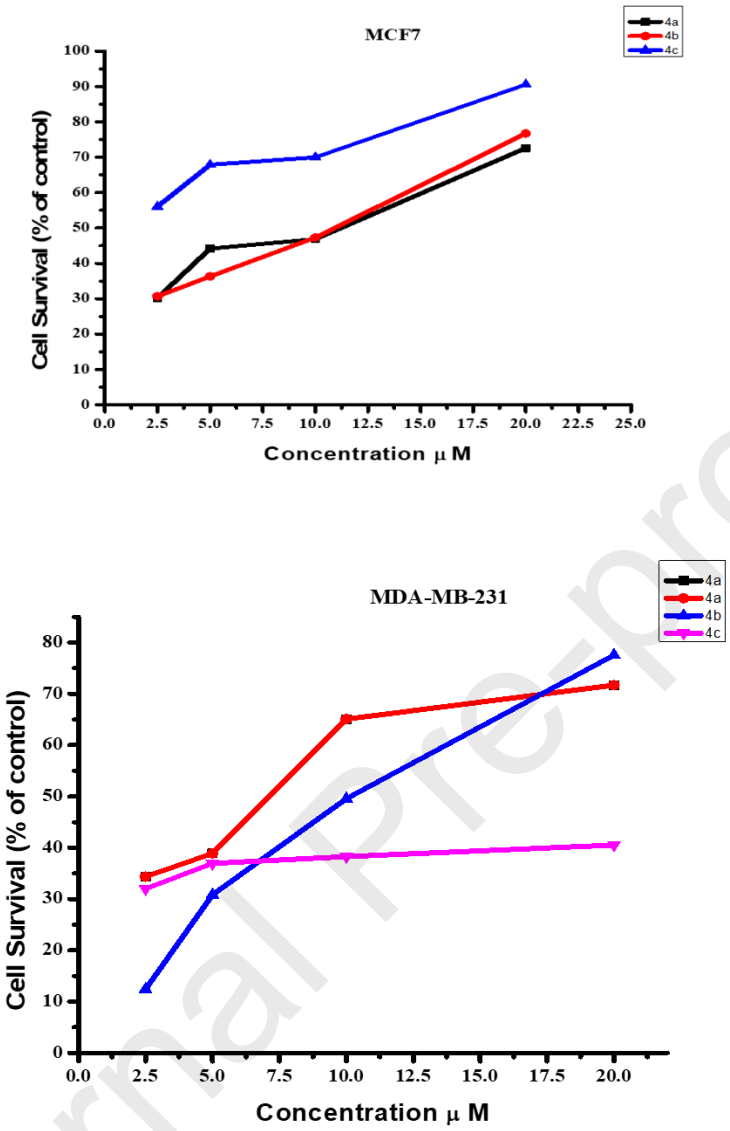


Fig. 5. Line graph showing the effect of compounds **4a-c** on the survival of MCF-7 and MDA-MB-231 human breast cancer cells

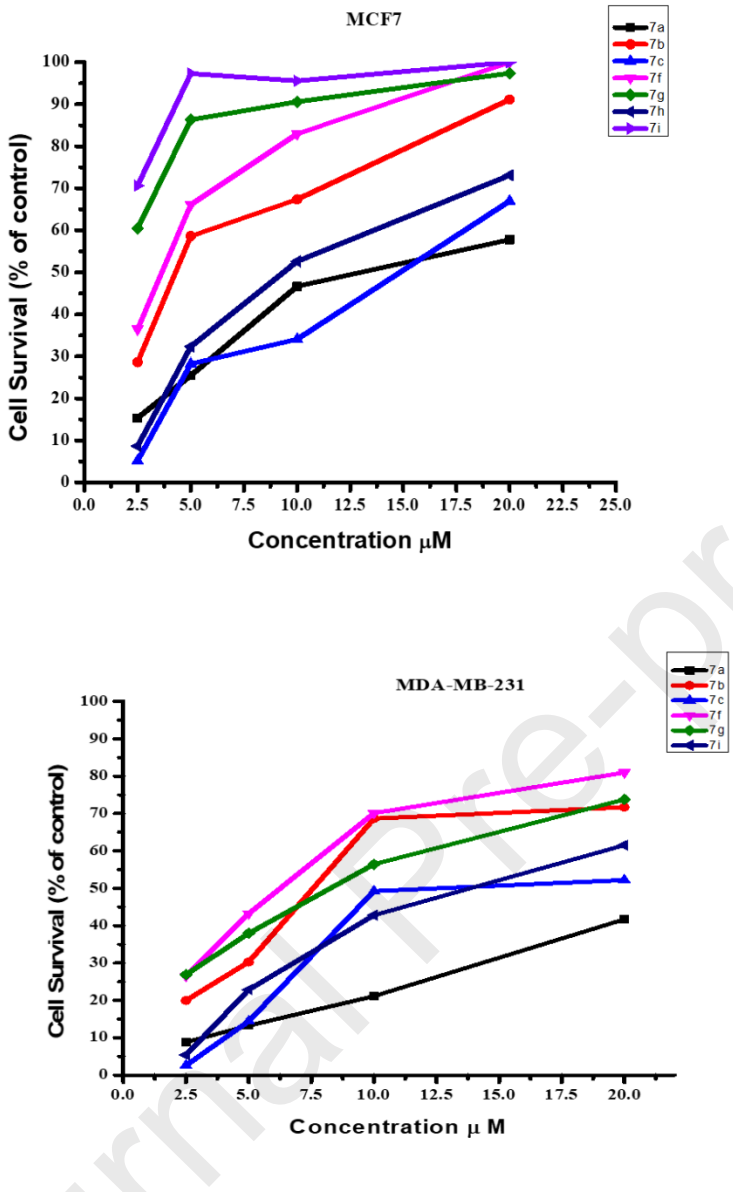


Fig. 6. Line graph showing the effect of a compound from series **7a-i** on the survival of MCF-7 and MDA-MB-231 human breast cancer cells

2.2.2. The s- triazine *derivatives caused significant degree of developmental toxicity in zebrafish embryos*

For preliminary toxicity tests, the zebrafish is a powerful drug screening model due to its rapid embryonic development, transparent embryos and feasibility of testing compounds in a small volume (due to small size of embryo) [32-39].

The zebrafish embryo toxicity screening assays revealed that most of the triazine derivatives were toxic. In this regard, at concentrations over 5 μM, all the derivatives caused 100% lethality (Table 2 and 3). However, as in the cancer cell lines, **7e** and **7d** showed no activity in these assays.

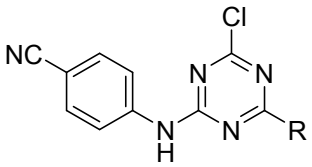
Compounds **3** and **6** showed a moderate level of toxicity in zebrafish embryos. The LD_{50} value (concentration at which half the treated embryos died) for **3** was 20 ± 0.25 μM, while for **6** it was 15± 0.20 μM. Zebrafish embryos treated with a sub-lethal concentration (below the LD_{50} values) of **3** showed developmental abnormalities. As shown in the photomicrograph in Table 2, the embryos treated with **3** were smaller than control embryos, thereby indicating that the compound impaired proliferation and development. Embryos treated with **6** showed cardiac edema and hypertrophy at concentrations ≥ 3 μM, as shown in the photomicrograph in Table 2.

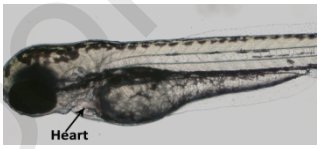
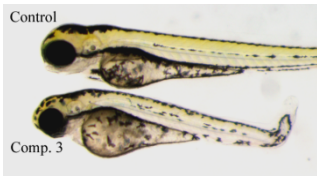
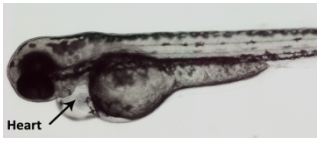
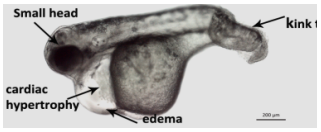
Compounds **7e** and **7d** showed no activity and were well tolerated by the zebrafish embryos. Indeed, no mortalities or embryonic abnormalities were observed even at a concentration of 40 μM.

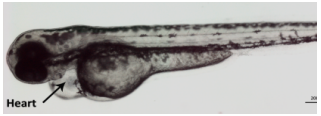
The remaining compounds showed a range of teratogenic effects at sub-lethal concentrations and the severity of the abnormalities directly correlated with the concentration of the compounds used. The results of the zebrafish screening assays with compounds **4a-c** and **7a-i** are summarized in Table 2 and 3, respectively. Almost all the compounds tested caused cardiac hypertrophy (larger heart), as shown in the accompanying photomicrograph in Table 2 and 3.

The effect of the *s*-triazine derivatives was consistent with the effects of treating zebrafish embryos with estrogen disruptors, such as bisphenol A [40,41]. The cardiac hypertrophy, cardiac edema, and bending of the tail by *s*-triazine derivatives observed in this study also resemble the phenotype of zebrafish embryo exposed to benomyl [42].

Table 2. The bioactivity of compounds **3, 4a-c** and **6** in zebrafish embryos

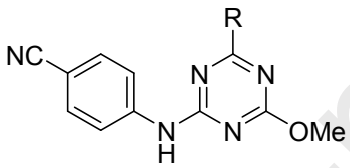


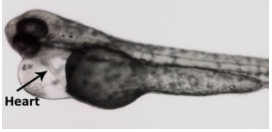
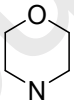
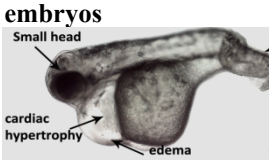
Comp ID	R	Concentration *			Representative micrograph of zebrafish embryos
		1 µM	**3 µM**	**5 µM**	
Control					Control 
3	Cl	**Not Toxic**	**Not Toxic**	**Not Toxic**	 The bodies of treated embryos were much smaller than those of controls and they showed cardiac edema.
6	OMe	Not active	Not Active	**Not toxic**	 The treated embryos had cardiac edema and cardiac hypertrophy
4a		**Not Toxic**	**Toxic** 100% mortality	**Toxic** 100% mortality	3 µM-treated embryos  The embryos treated with 0.5 µM had short bodies, smaller heads, kinked tails, cardiac hypertrophy and severe developmental delay

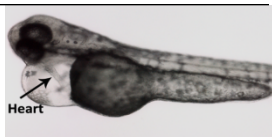
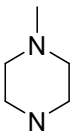
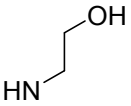
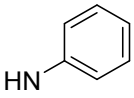
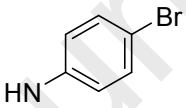
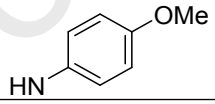
4b		Not Active	Not Active	Not Toxic	 3 µM-treated embryos showed cardiac edema and cardiac hypertrophy
4c		**Not Toxic**	**Toxic** 100% mortality	**Toxic** 100% mortality	**Same as 4a**

* Three biological replicates were done for each treatment, using at least 30 embryos in each treatment

Table 3. The bioactivity of compounds **7a-i** in zebrafish embryos



Compd ID	**R**	**Concentration used ***			**PHENOTYPE**
		0.5 µM	**1 µM**	**3 µM**	
Control		-	-	-	
7a		**Not toxic**	**Not toxic**	**Toxic** 100% mortality	 3 µM-treated embryos showed severe cardiac hypertrophy and cardiac edema
7b		**Not toxic**	**Toxic** 100% mortality	**Toxic** 100% mortality	**3 µM-treated embryos**  had short bodies, smaller heads, kinked tails and cardiac hypertrophy

7c		**Not toxic**	**Not toxic**	**Toxic** 100% mortality	 Cardiac hypertrophy
7d		**Not active**	**Not active**	**Not active**	
7e		**Not active**	**Not active**	**Not active**	
7f		**Not toxic**	**Toxic** 100% mortality	**Toxic** 100% mortality	**Same as 7b**
7g		**Not toxic**	**Toxic** 100% mortality	**Toxic** 100% mortality	**Same as 7b**
7h		**Not toxic**	**Not toxic**	**Toxic** 100% mortality	**Same as 7a**
7i		**Not toxic**	**Toxic** 100% mortality	**Toxic** 100% mortality	**Same as 7b**

* Three biological replicates for each treatment were done, using at least 30 embryos in each treatment

3. Conclusion

The newly synthesized s-triazine derivatives reported herein showed strong anticancer activity against hormone receptor-positive human breast cancer cell line MCF-7 and triple receptor negative breast cancer cell line MDA-MB-231. In this regard, compounds **7i** and **7g** had IC_{50} values of less than 1 μM. Moreover, these compounds showed selective cytotoxicity in MCF-7 cells. Most of the trisubstituted *s*-triazine derivative **7** series showed higher activity than the disubstituted **4** series. While Compound **4c** showed high selectivity for MCF-7 cells (IC_{50} 1.3 ± 0.01). The combination between the methoxy group, 4-aminobenzonotrile and aniline ring in the triazine core showed the highest activity, with **7g** and **7i** showing IC_{50} values of 0.77 ± 0.01 and 0.1 ± 0.01 μM for the MCF-7 cell line and 8.43 ± 0.01 and 14.28 ± 0.01 μM for the MDA-MB-231 cell line, respectively. These two compounds have two aniline moieties, and it appears that the presence of electron-donating substituents (OCH_3) (**7i**) is relevant for the activity. Compound **7h** with a bromoaniline moiety showed much less activity, presumably due to both the electron-withdrawing effect and the hindrance of the Br. Compared with the other derivatives, which had only one aniline, in addition to the 4-aminobenzonitrile group; IC_{50} values for **7b** and **7f** were 4.29 ± 0.02 and 3.71 ± 0.04 μM in the MCF-7 cell line, respectively, and 7.80 ± 0.03 and 6.49 ± 0.04 μM, respectively in the MDA-MB-231 cell line. In contrast, **7d** and **7e** were completely inactive in both cell lines. In addition the combination between the methoxy and the morpholino group beside the 4-aminobenzonitrile derivative behaves in the same manner, where it showed higher activity than the piperidino analogs, in agreement with previous reported data [20].

The zebrafish embryo screening assays revealed that compounds from series **4a-c** and **7a-i** induced cardiac hypertrophy. The effect of the compounds reported herein correlate with published reports of zebrafish embryos treated with estrogen disruptors such as bisphenol A [40,41]. The cardiac hypertrophy, cardiac edema, and bending of the tail induced by *s*-triazine derivatives reported here are also similar to the phenotype of zebrafish embryos exposed to benomyl [42].

Overall, given that **7i** was the most active compound and showed toxicity only at 1 μM, thus having a therapeutic window of 10, it deserves further attention as a potential HIT.

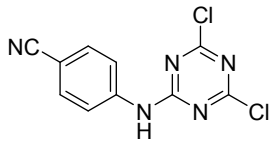
Finally, this work has demonstrated once again that the triazine core is a privileged structure that should be considered in medicinal chemistry programs.

4. Experimental

4.1. Chemistry

Material and Methods: All reagents and solvents were obtained from commercial suppliers and used without further purification, ^{1}H-NMR and ^{13}C-NMR were recorded on a JEOL 400 MHz spectrometer, the chemical shifts (δ) are referred in terms of *ppm* and *J* -coupling constants are given in *Hz*. Elemental analysis was carried out on an Elmer 2400 Elemental Analyzer. All melting points for the prepared compounds were measured on a Gallenkamp melting point apparatus in open glass capillaries and are uncorrected.

*4.1.1. Synthesis of 4-((4,6-dichloro-1,3,5-triazin-2-yl)amino)benzonitrile , **3** [25]*

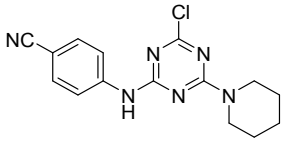


A solution of 4-aminobenzonitrile **2** (1.84 g, 0.01 mol) was added portion-wise to a mixture of cyanuric chloride **1** (1.85 g, 0.01 mol) and anhydrous potassium carbonate (K_2CO_3) (1.38 g, 0.01 mol) in 25 ml acetone at 0–5 ^{o}C over 15 min. After the addition, the reaction mixture was stirred for 4 h at the same temperature. TLC using MeOH/Chloroform (2:8) was used to monitor the progress of the reaction. After completion of the reaction, the reaction mixture was poured onto crushed ice. The solid product obtained was filtered, washed with distilled water, dried, and purified or used directly for the next step. White solid; Yield 92%; m.p. 288-290^{o}C [Lit. [25] 300^{o}C (dec.)]; ^{1} H-NMR (DMSO-d_6): δ 11.50 (s, 1H, –NH proton of s-triazine to aminobenzonitrile linkage), 7.86–7.78 (m, 4H, Ar–H aromatic proton); ^{13}C-NMR (DMSO- d_6): δ 106.84 (–CN of aminobenzonitrile moiety), 141.99–119.32 (6C, Ar–C of aromatic), 164.37 (C–NH of s-triazine to aminobenzonitrile linkage), 173.81 (C–Cl of s-triazine).

4.1.2. General method for the synthesis of 4-chloro-6-subsitited (1,3,5-triazin-2-yl)aminobenzonitrile

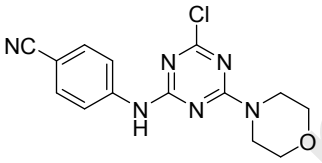
A solution of the amine (10 mmole) in 50 mL THF was added dropwise to a solution of 4,6-dichloro (1,3,5-triazin-2-yl)aminobenzonitrile (10 mmol) and K_2CO_3 (10 mmol) in 50 mL THF over 5 min. The reaction mixture was stirred at rt for 24 h and then THF was concentrated under vacuum and excess ice water was added. The product was obtained as a white solid. It was then filtered, washed with water and dried. The crude product was recrystallized from ethylacetate.

*4.1.2.1. 4-((4-Chloro-6-(piperidin-1-yl)-1,3,5-triazin-2-yl)amino)benzonitrile, **4a***



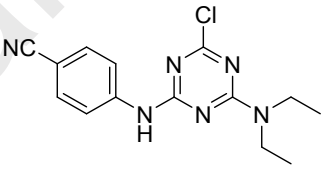
White solid; Yield 85%; m.p. 164-165°C; ^{1}H-NMR (DMSO-d_6) δ: 1.53-1.62 (m, 6H, 3CH_2), 3.69-3.74 (m, 4H, 2CH_2), 7.75(d, 2H, *J* = 8.8 Hz, Ar), 7.82 (d, 2H, *J* = 8.8 Hz, Ar), 10.44(br.s., 1H, NH) ppm; ^{13}C-NMR(DMSO-d_6): δ 23.9, 25.3, 66.9, 104.2,119.2, 119.8, 133.1, 143.4, 163.4, 168.8, 175.5 ppm. Anal.Calc for $C_{15}H_{15}ClN_6$ (314.78): C, 57.24; H, 11.26; N, 26.70. Found: C, 57.45; H, 11.41; N, 26.86.

4.1.2.2. 4-((4-Chloro-6-morpholino-1,3,5-triazin-2-yl)amino)benzonitrile, ***4b***



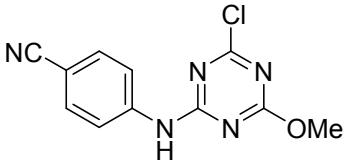
White solid; Yield 84%; m.p. 238-240°C; ^{1}H-NMR (DMSO-d_6) δ: 3.64 (t, 4H, *J* = 8.0 Hz, CH_2-N-CH_2), 3.74 (t, 4H, *J* = 8.0 Hz, CH_2-O-CH_2), 7.75(d, 2H, *J* = 8.8 Hz, Ar), 7.84 (d, 2H, *J* = 8.8 Hz, Ar), 10.53(br.s., 1H, NH) ppm; ^{13}C-NMR(DMSO-d_6): δ 43.9, 65.7, 104.4 (CN), 119.1, 119.5, 120.1, 132.9, 133.3, 143.2, 163.5, 163.9, 168.6, 175.5 ppm. Anal.Calc for $C_{14}H_{13}ClN_6O$ (316.75): C, 53.09; H, 11.19; N, 26.53. Found: C, 53.25; H, 11.33; N, 26.87.

4.1.2.3. 4-((4-Chloro-6-(diethylamino)-1,3,5-triazin-2-yl)amino)benzonitrile, ***4c***



White solid;Yield 88%; m.p. 142-144 °C; ^{1}H-NMR (DMSO-d_6) δ: 1.09-1.18 (m 6H, 2CH_3), 3.51-3.59 (m, 4H, 2CH_2), 7.74(d, 2H, *J* = 8.8 Hz, Ar), 7.88 (d, 2H, *J* = 8.8 Hz, Ar), 10.46(s, 1H, NH) ppm; ^{13}C-NMR(DMSO-d_6): δ 12.8, 12.9,41.5,41.9, 104.1, 119.2, 119.7, 133.1, 143.5, 163.3, 168.3, 175.5 ppm. Anal.Calc for $C_{14}H_{15}ClN_6$ (302.77): C, 55.54; H, 4.99; N, 27.76. Found: C, 55.78; H, 5.13; N, 27.99.

4.1.2.4. Synthesis of 4-chloro-6-methoxy(1,3,5-triazin-2-yl)amino)benzonitrile, ***6***

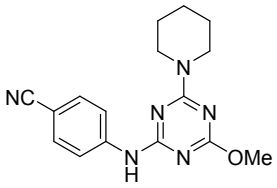


A solution of 4-aminobenzonitrile (10 mmole) in 50 mL acetone was added dropwise, as described previously [32], to an ice-cold solution of 2,4-dichloro-6-methoxy-1,3,5-triazine **5** (10 mmol) and $NaHCO_3$ (10 mmol) in 50 mL acetone over 10 min. The reaction mixture was stirred at rt for 24 h and then acetone was concentrated under vacuum, and excess ice water was added. The solid product obtained was filtered, washed with water, and dried to afford the product. White solid; Yield 87%; m.p. 226-227 ^oC; ^1H-NMR ($CDCl_3$) δ: 4.06 (s, 3H, OCH_3), 7.49 (br.s., 1H, NH), 7.64(d, 2H, *J* = 8.8 Hz, Ar), 7.72 (d, 2H, *J* = 8.8 Hz, Ar) ppm; ^{13}C-NMR ($CDCl_3$) δ: 52.1 (OMe), 102.2 (CN), 120.3, 133.4, 163.5, 172.3 ppm. Anal.Calc for $C_{11}H_8ClN_5O$ (261.67): C, 50.49; H, 3.08; N, 26.76. Found: C, 50.66; H, 3.19; N, 26.92.

4.1.3. General method for the synthesis of 4-substituted-6-methoxy((1,3,5-triazin-2-yl) aminobenzonitrile

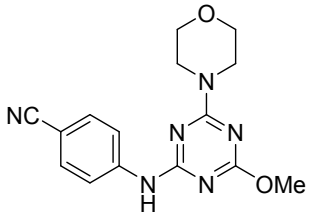
A solution of 4-amine (10 mmole) in 50 mL acetonitrile was added dropwise to a solution of 4-chloro-6-methoxy(1,3,5-triazin-2-yl)amino)benzonitrile (10 mmol) and K_2CO_3 (10 mmol) in 50 mL acetonitrile, and the reaction mixture was stirred under reflux 18 h. Acetonitrile was then concentrated under vacuum, and excess ice water was added. The product was obtained as white solid. It was filtered, washed with water and dried. The crude product was recrystallized from ethylacetate-ethanol (3:1)

*4.1.3.1. 4-((4-Methoxy-6-(piperidin-1-yl)-1,3,5-triazin-2-yl)amino)benzonitrile, **7a***



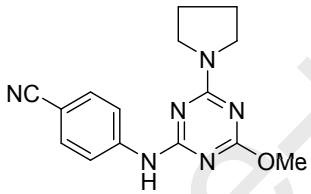
White solid; Yield 82%; m.p. 213-214 ^oC; ^1H-NMR ($CDCl_3$) δ: 1.59 (br.s., 4H, $2CH_2$), 1.67 (br.s., 2H, CH_2), 3.78 (br.s. 4H, $2CH_2$), 3.92(s, 3H, OCH_3), 7.57(d, 2H, *J* = 8.8 Hz, Ar), 7.72 (d, 2H, J = 8.8 Hz, Ar), 7.82(br.s., 1H, NH) ppm; ^{13}C-NMR ($CDCl_3$): δ 24., 25.7, 44.9, 54.3, 105.2, 119.2, 119.5, 133.0, 143.1, 164.3, 164.9, 170.4 ppm. Anal.Calc for $C_{16}H_{18}N_6O$ (310.36): C, 61.92; H, 5.85; N, 27.08. Found: C, 62.15; H, 5.72; N, 27.29.

*4.1.3.2. 4-((4-Methoxy-6-morpholino-1,3,5-triazin-2-yl)amino)benzonitrile, **7b***



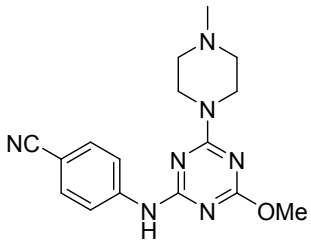
White solid; Yield 84%; m.p. 216-218 °C; ^{1}H-NMR ($CDCl_3$) δ: 3.73(br.s., 4H, $2CH_2$), 3.83(br.s., 4H, $2CH_2$), 3.94 (s. 3H, OCH_3), 7.56(d, 2H, J = 8.8 Hz, Ar), 7.70 (d, 2H, *J* = 8.4 Hz, Ar), 10.22(s., 1H, NH) ppm; ^{13}C-NMR ($CDCl_3$): δ 44.5, 54.2, 66.6, 105.3, 119.3, 119.6, 122.3, 133.1, 142.7, 164.3, 165.6, 170.4, ppm. Anal.Calc for $C_{15}H_{16}N_6O_2$ (312.33): C, 57.68; H, 5.16; N, 26.91. Found: C, 57.76; H, 5.33; N, 27.13.

4.1.3.3. 4-((4-Methoxy-6-(pyrrolidin-1-yl)-1,3,5-triazin-2-yl)amino)benzonitrile, ***7c***



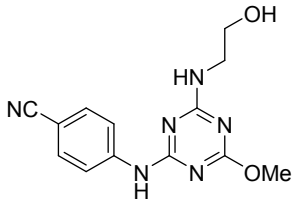
White solid; Yield 86%; m.p. 223-25 °C; ^{1}H NMR ($CDCl_3$) δ: 1.96-2.02 (m, 4H, $2CH_2$), 3.60-3.64(m, 2H, CH_2), 3.95 (s. 4H, OCH_3), 7.57(d, *J* = 8.8, 2H, Ar), 7.76 (d, 2H, *J* = 8.8 Hz, Ar), 8.67(br.s., 1H, NH) ppm; ^{13}C-NMR ($CDCl_3$): δ 24.9, 25.0, 46.5, 46.7, 54.2, 105.2, 119.1, 119.4, 132.8, 143.1, 164.3, 164.9, 170.4 ppm. Anal.Calc for $C_{15}H_{16}N_6O$ (296.33): C, 60.80; H, 5.44; N, 28.36. Found: C, 60.98; H, 5.65; N, 28.59.

4.1.3.4. 4-((4-Methoxy-6-(4-methylpiperazin-1-yl)-1,3,5-triazin-2-yl)amino)benzonitrile, ***7d***



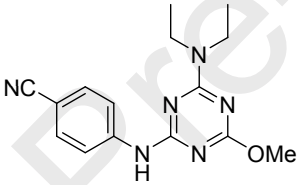
White solid; Yield 87%; m.p. 186-187 °C; ^{1}H-NMR ($CDCl_3$) δ: 2.19 (s, 3H, NCH_3), 2.34 (br.s., 4H, $2CH_2$), 3.78 (br.s. 4H, $2CH_2$), 3.84(s, 3H, OCH_3), 7.70(d, 2H, *J* = 8.8 Hz, Ar), 7.90 (d, 2H, *J* = 8.8 Hz, Ar), 10.02(s, 1H, NH) ppm; ^{13}C-NMR ($CDCl_3$): δ 42.8, 45.7, 54.8,54.2, 103.3, 119.3, 119.4, 132.9, 144.2, 165.0, 165.2, 170.6,175.5 ppm. Anal.Calc for $C_{16}H_{19}N_7O$ (325.38): C, 59.06; H, 5.89; N, 30.13. Found: C, 59.33; H, 6.03; N, 30.43.

4.1.3.5. 4-((4-((2-Hydroxyethyl)amino)-6-methoxy-1,3,5-triazin-2-yl)amino)benzonitrile, ***7e***



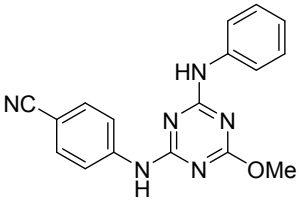
White solid;Yield 80%; m.p. 190-192 ^{o}C; ^{1}H-NMR ($CDCl_3$) δ: 3.37 (m, 2H,), 3.52 (m, 2H, CH_2), 3.78 (s, 3H, OCH_3), 4.71(m,1H, OH), 7.58-7.72(m, 3H, NH, Ar), 7.99(dd, 2H, *J* = 8.0, 8.8 Hz, Ar), 9.99 (s, 1H, NH) ppm; ^{13}C-NMR ($CDCl_3$): δ 42.9, 53.7, 59.5, 103.1, 119.4, 132.9, 144.4, 164.7, 166.7, 170.4, 175.5 ppm. Anal. Calc for $C_{13}H_{14}N_6O_2$ (286.30): C, 54.54; H, 4.93; N, 29.36. Found: C, 54.79; H, 4.86; N, 29.58.

4.1.3.6. *4-((4-(Diethylamino)-6-methoxy-1,3,5-triazin-2-yl)amino)benzonitrile,* ***7f***



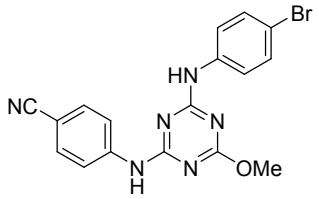
White solid; Yield 89%; m.p. 182-183 ^{o}C; ^{1}H-NMR ($CDCl_3$) δ: 1.17-1.24(m, 6H, $2CH_3$), 3.57-3.64(m, 4H, $2CH_2$), 3.92 (s, 3H, OCH_3), 7.56(d, 2H, *J* = 6.8 Hz, Ar), 7.70 (d, 2H, *J* = 6.8 Hz, Ar), 7.82(s., 1H, NH) ppm; ^{13}C-NMR ($CDCl_3$): δ 13.1, 41.2, 54.2, 66.6, 105.2, 119.3, 132.9, 143.2, 164.1, 165.0, 170.2, ppm. Anal. Calc for $C_{15}H_{18}N_6O$ (298.35): C, 60.39; H, 6.08; N, 28.17.

4.1.3.7. 4-((4-Methoxy-6-(phenylamino)-1,3,5-triazin-2-yl)amino)benzonitrile, ***7g***



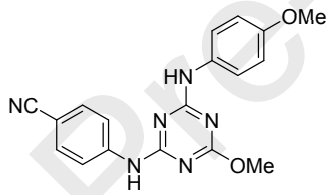
White solid; Yield 83%; m.p. 256-258 ^{o}C; ^{1}H-NMR (DMSO-d_6) δ: 3.91 (s. 3H, OCH_3), 7.01(m, 1H, Ar), 7.31-7.37(m, 3H, Ar), 7.72(d, 4H, *J* = 8.8 Hz, Ar), 7.99 (br.s., 2H, Ar), 9.85 (s, 1H, NH) 10.15(s, 1H, NH) ppm; ^{13}C-NMR (DMSO-d_6): δ 54.2, 103.6, 119.3, 119.7, 120.2, 120.8, 122.9, 128.5, 128.8, 132.9, 139.1, 144.1, 165.2, 170.7, 175.5 ppm. Anal.Calc for $C_{17}H_{14}N_6O$ (318.34): C, 64.14; H, 4.43; N, 26.40. Found: C, 64.37; H, 4.66; N, 26.69.

4.1.3.8. 4-((4-((4-Bromophenyl)amino)-6-methoxy-1,3,5-triazin-2-yl)amino)benzonitrile, ***7h***



White solid; Yield 81%; m.p. 258-260 ^{o}C; ^{1}H-NMR (DMSO-d_6) δ: 3.90 (s. 3H, OCH_3), 7.50(d, 2H, J = 8.4 Hz, Ar), 7.73 (d, 2H, *J* = 8.4 Hz, Ar), 7.87-7.96 (m, 4H, AR), 10.0 (s., 1H, NH) 10.22(s., 1H, NH) ppm; ^{13}C-NMR (DMSO-d_6): δ 54.2, 103.8, 105.2, 114.5,119.4, 119.9, 122.3, 131.4, 133.2, 142.5, 143.9, 165.0, 165.0, 170.4, 175.5 ppm. Anal.Calc for $C_{17}H_{13}BrN_6O$ (397.24): C, 51.40; H, 3.30; N, 21.16. Found: C, 51.67; H, 3.43; N, 21.39.

4.1.3.9. 4-((4-Methoxy-6-((4-methoxyphenyl)amino)-1,3,5-triazin-2-yl)amino) benzonitrile, ***7i***



White solid; Yield 86%; m.p. 206-208^{o}C; ^{1}H-NMR (DMSO-d_6) δ: 3.73(s, 3H, OCH_3), 3.91 (s. 3H, OCH_3), 6.89(d, 2H, *J* = 8.8 Hz, Ar), 7.57(br.s., 2H, Ar), 7.72(d, 2H, *J* = 8.8 Hz, Ar), 7.92(br.s.,2H, Ar), 9.70 (s, 1H, NH) 10.10(s, 1H, NH) ppm; ^{13}C-NMR (DMSO-d_6): δ 54.1, 55.2, 103.5, 113.7, 119.4, 119.6, 131.9, 132.9, 144.2, 155.3, 165.1, 170.6, 175.5 ppm. Anal. Calc for $C_{18}H_{16}N_6O_2$ (348.37): C, 62.06; H, 4.63; N, 24.12. Found: C, 62.30; H, 4.77; N, 24.37.

4.2. Biology

4.2.1. Zebrafish embryo treatment.

The zebrafish embryos were obtained by natural pair wise breeding and treated essentially as described previously [43]. The compounds were dissolved in dimethyl sulfoxide (DMSO) to prepare a stock concentration of 10 mM. Embryos treated with 0.10% DMSO (v/v) served as negative controls.

4.2.2. Imaging and microscopy.

A Zeiss Observer D1 inverted microscope was used to capture images of live zebrafish embryos using Zeiss ZEN software.

4.2.3. MTT cell proliferation assays

Human breast adenocarcinoma cells MCF-7 (ATCC® HTB-22™) and MDA-MB-231 (ATCC® HTB-26™) were from the American Type Culture Collection (American Type Culture Collection (ATCC) Manassas, VA 20108 USA). The cells were maintained in Dulbecco's Modified Eagle's Medium DMEM (11965-092 Thermo Fisher supplemented with 10% FCS (Cambrex Bio Science), 100 IU/mL penicillin, 100 mg/mL streptomycin and 2 mmol/L L-glutamine (Sigm-Aldrich). The cells were supplemented with 10% fetal bovine serum (Lonza) and 1% ABM (GIBCO) and maintained at37˚C and 5% CO_2 in a humidified cell culture incubator.

4.2.4. Cell culture and cell viability assay

The MCF-7 and MDA-MB-231 lineswere grown in 48-well culture plates. They were allowed to adhere to the plate for 6 h and allowed to grow for 24 h. The cells were treated with serial dilution of each compound (in triplicate) and treated for 24 h. Cytotoxicity was tested using a colorimetric MTT cell viability assay, which was performed essentially as described previously [44].

4.2.5. Statistics

The zebrafish screening assays were done in three biological replications using embryos from different batches of adult fish each time. Replication was done with at least 30 embryos. The cell proliferation assay in human breast cancer cell lines was done at five concentrations of the compounds (0.5, 1, 2, 4, and 10 μM) and triplicate 48-well culture plates. Origin pro 8.5 was used to calculate the IC_{50} values and standard deviation in the MTT assay.

Acknowledgements

The work was funded in part by the following: the Deanship of Scientific Research at King Saud University Research Group no. RGP-234, Saudi Arabia; the National Research Foundation (NRF) (Blue Sky's Research Programme # 110960) and the University of KwaZulu-Natal (South Africa); and the Spanish Ministry of Science, Innovation, and Universities (CTQ2015-67870-P) and the Generalitat de Catalunya (2017 SGR 1439; Spain).

References

[1] B. Zacharie, S.D. Abbott, J.S. Duceppe, L. Gagnon, B. Grouix, L. Geerts, L. Gervais, F. Sarra-Bournet, V. Perron, N. Wilb, C.L. Penney, and P. Laurin, ChemistryOpen 7 (2018) 737-749.

[2] A. Sharma, A. El-Faham, B.G. de la Torre, and F. Albericio, Frontiers in Chemistry 6 (2018) 516.

[3] I.G. Salado, A. Baan, T. Verdeyen, A. Matheeussen, G. Caljon, P. Van der Veken, F. Kiekens, L. Maes, and K. Augustyns, Eur. J. Med. Chem. 151 (2018) 18-26.

[4] L.M. Moreno, J. Quiroga, R. Abonia, J. Ramirez-Prada, and B. Insuasty, Molecules 23 (2018) 1956.

[5] M.J. Akhtar, A.A. Khan, Z. Ali, R.P. Dewangan, M. Rafi, M.Q. Hassan, M.S. Akhtar, A.A. Siddiqui, S. Partap, S. Pasha, and M.S. Yar, Bioorganic Chemistry,78 (2018) 158-169.

[6] E.S. Kim and Enasidenib, Drugs 77(2017) 1705-1711.

[7] R. Lin, G. Chiu, Y. Yu, P.J. Connolly, S. Li, Y. Lu, M. Adams, A.R. Fuentes-Pesquera, S.L. Emanuel, and L.M. Greenberger, Bioorg.Med. Chemistry Lett., 17(16) (2007) 4557-61.

[8] N. Baindur, N. Chadha, B.M. Brandt, D. Asgari, R.J. Patch, C. Schalk-Hihi, T.E. Carver, I.P. Petrounia, C.A. Baumann, H. Ott, C. Manthey, B.A. Springer, and M.R. Player, J. Med. Chem. 48 (2005) 1717-20.

[9] M.J.F. Calvete, S.M.A. Pinto, H.D. Burrows, M.M.C.A. Castro, C.F.G.C. Geraldes, and M.M. Pereira, Arab. J. Chem. (2018); https://doi.org/10.1016/j.arabjc.2018.06.005

[10] G. Blotny, Tetrahedron 62 (2006) 9507-9522.

[11] S. Nozaki, M. Maeda, H. Tsuda, G. W. Sledge, Breast Cancer Res. Treat. 83 (2004) 195-199.

[12] F. Saczewski, A. Bulakowska, P. Bednarski, and R. Grunert, Eur. J. Med. Chem. 41 (2006) 219-225.

[13] A. Agarwal, K. Srivastava, S. K. Puri, P. M. S. Chauhan, Bioorg. Med. Chem. Lett. 15 (2005) 53-533.

[14] A. Solankee, K. Kapadia, A. Ciric, M. Sokovic, I. Doytchinova, and A. Geronikaki, Eur. J. Med. Chem. 45 (2010) 510-518.

[15] R. V. Patel, P. Kumari, D. P. Rajani, C. Pannecouque, E. De Clercq, and K. H. Chikhalia, Future Med. Chem. 4 (2012) 1053-1065.

[16] R.V. Patel, P. Kumari, D.P. Rajani, and K.H. Chikhalia, J. Fluorine Chem. 132 (2011) 617–627.

[17] R.V. Patel, P. Kumari, D.P. Rajani, and K.H. Chikhalia, Eur. J. Med. Chem. 46 (2011) 4354–4365.

[18] N. Sunduru, L. Gupta, V. Chaturvedi, R. Dwivedi, S. Sinha, and P.M. Chauhan, Eur. J. Med. Chem. 45 (2010) 3335–3345.

[19] A. El-Faham and Y.A. Elnakady, Lett. Org. Chem., 12 (2015) 753-758.

[20] A. El-Faham, S.M. Soliman, H.A. Ghabbour, Y.A. Elnakady, T.A. Mohaya, M.R.H. Siddiqui, and F. Albericio, J. Mol. Str., 1125 (2016) 121-135.

[21] M. Farooq, A. Sharma, Z. Almarhoon, A. Al-Dhfyan, A. El-Faham, N.Abu Taha, M. A. M. Wadaan, B. G. de laTorre, and F. Albericio, Bioorganic Chemistry 87 (2019) 457–464.

[22] P.M. Eimon and A.L. Rubinstein, Expert Opin Drug Met. 5 (2009) 393-401.

[23] U. Strahle, S. Scholz, R. Geisler, P. Greiner, H. Hollert, S. Rastegar, A. Schumacher, I. Selderslaghs, C. Weiss, H. Witters, and T. Braunbeck, Reprod. Toxicol. 33 (2012) 128-132.

[24] A. El-Faham, S.M. Osman, H.A. Al-Lohedan, and G.A. El-Mahdy, Molecules 21(2016) 714.

[25] P.K. Patel, R.V. Patel, D.H. Mahajan, P.A. Parikh, G.N. Mehta, C. Pannecouque, E. De Clercq, and K.H. Chikhalia, J. Heterocyclic Chem. 51(2014) 1641-1658.

[26] R.M. Abdelrahman, M. Seada, M. Fawzy, and I. Elbaz, Pharmazie 49 (1994) 811-814.

[27] I.M. Labouta, N.H. Eshba, and H.M. Salama, Monatsh Chem 119 (1988) 591-596.

[28] W. Huang, W. Zheng, D. J. Urban, J. Inglese, E. Sidransky, C. P. Austin and C. J. Thomas, Bioorg. Med. Chem.Lett.17 (2007) 5783–5789.

[29] M. Venkatraj , K. K. Ariën, J. Heeres, J. Joossens , B. Dirié a, S. Lyssens, J. Michiels , P. Cos , P. J. Lewi, , G. Vanham, L. Maes , P. Van der Veken , K.Augustyns . Bioorg. Med. Chem. 22 (19) (2014) 5241-5248.

[30] X. Dai, H. Cheng, Z. Bai, and J. Li, J. Cancer 8 (2017) 3131-3141.

[31] G.J. Kumar, S.N. Kumar, D. Thummuri, L.B.S. Adari, V.G.M. Naidu, K. Srinivas, and V.J. Rao, Med. Chem. Res. 24 (2015) 3991-4001.

[32] H. Araujo-Silva, J. Pinheiro-da-Silva, P.F. Silva, and A.C. Luchiari, PloS One 13 (2018)1-12.

[33] M.F.J. Arief, B.K.M. Choo, J.L. Yap, Y. Kumari, and M.F. Shaikh, Frontiers Pharmacol. 9 (2018).

[34] J. Byrnes, R. Ganetzky, R. Lightfoot, M. Tzeng, E. Nakamaru-Ogiso, C. Seiler, and M.J. Falk, Neurochem. Int. 117 (2018) 23-34.

[35] C. Cornet, V. Di Donato, and J. Terriente, Frontiers Pharmacol. 9 (2018) 703. doi: 10.3389/fphar.2018.00703

[36] J. Gehrig, G. Pandey, and J.H. Westhoff, Frontiers Pediatr. 6 (2018) 183. doi: 10.3389/fped. 2018.00183.

[37] A. Griffin, K.R. Hamling, S. Hong, M. Anvar, L.P. Lee, and S.C. Baraban, Frontiers Pharmacol 9 (2018) 573.

[38] Y. Muniandy, Zebrafish 15 (2018) 321-339.

[39] R.L. Vaz, T.F. Outeiro, and J.J. Ferreira, Frontiers Neurol. 9 (2018) 347. doi: 10.3389/fneur.2018.00347

[40] A. Hamad, M. Kluk, J. Fox, M. Park, and J.E. Turner, Current Eye Res. 32 (2007) 819-27.

[41] W.K. Tse, B.H. Yeung, H.T. Wan, and C.K. Wong, BiologyOpen 2 (2013) 466-71.

[42] D.J. Kim, S.H. Seok, M.W. Baek, H.Y. Lee, Y.R. Na, S.H. Park, H.K. Lee, N.K. Dutta, K. Kawakami, and J.H. Park, J. Appl. Toxicol : JAT 29

[43] M. Farooq, Z.M. Al Marhoon, N.A. Taha, A.A. Baabbad, M.A. Al-Wadaan, and A. El-Faham, Biological Pharm. Bull. 41 (2018) 350-359.

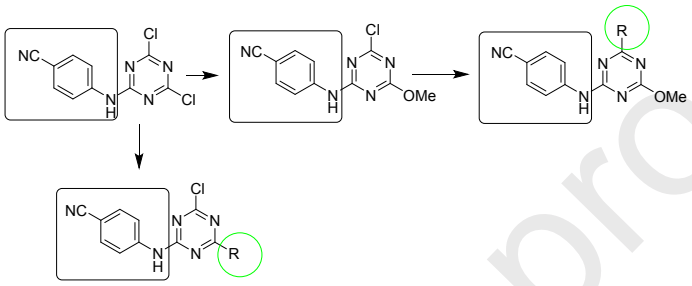
[44] M. Farooq, N.A. Taha, R.R. Butorac, D.A. Evans, A.A. Elzatahry, E.A. Elsayed, M.A. Wadaan, S.S. Al-Deyab, and A.H. Int. J. Mol. Sci.16 (2015) 24718-24731.

(2009) 289-94.

Graphical Abstract

Di- and tri-substituted s-triazine derivatives: Synthesis,characterization anticancer activity in human breast-cancer cell lines and developmental toxicity in zebrafish embryos

Ayman El-Faham*, Muhammad Farooq , Zainab Almarhoon, Rakia Abd Alhameed, Mohammad A.M. Wadaan, Beatriz G. de la Torre, Fernando Albericio *



Small library based on 4-aminobenzonitile-*s*-triazine moiety has been synthesized and fully characterized by ^{1}H-NMR, ^{13}C-NMR and elemental analysis. The reported *s*-triazine derivatives had shown strong anticancer activity against two of human breast cancer cell lines (MCF-7 and MIDA-MB-231) with IC_{50} values less than 1 μM. General trend that these compounds were more selective towards hormone receptor positive breast cancer cell line MCF-7 as compared to MDA-MB-231 cell line. Zebrafish embryos were used to evaluate *in vivo* and developmental toxicities of the target compounds.

Highlights

- Di-and trisubstituted-*s*-triazine based on 4-aminobenzonitrile moiety.
- Anticancer profile.
- Human breast carcinoma (MCF 7 and MDA-MB-231)
- *In vivo* toxicity in zebrafish embryos.