
# New Benzothieno[3,2-*d*]-1,2,3-triazines with Antiproliferative Activity: Synthesis, Spectroscopic Studies, and Biology Activity

Antonino Lauria, Alessia Alfio, Riccardo Bonsignore, Carla Gentile, Annamaria Martorana, Giuseppe Gennaro, Giampaolo Barone, Alessio Terenzi, Anna Maria Almerico

PII: S0960-894X(14)00635-0
DOI: http://dx.doi.org/10.1016/j.bmcl.2014.06.007
Reference: BMCL 21726

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 14 April 2014
Revised Date: 2 June 2014
Accepted Date: 4 June 2014

Please cite this article as: Lauria, A., Alfio, A., Bonsignore, R., Gentile, C., Martorana, A., Gennaro, G., Barone, G., Terenzi, A., Almerico, A.M., New Benzothieno[3,2-*d*]-1,2,3-triazines with Antiproliferative Activity: Synthesis, Spectroscopic Studies, and Biology Activity, *Bioorganic & Medicinal Chemistry Letters* (2014), doi: http://dx.doi.org/10.1016/j.bmcl.2014.06.007

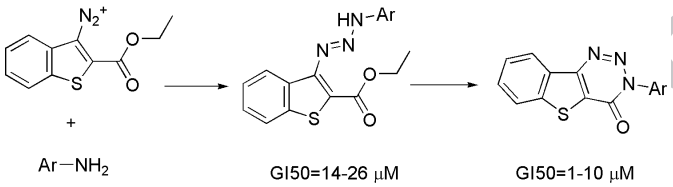
## Graphical Abstract

To create your abstract, type over the instructions in the template box below.
Fonts or abstract dimensions should not be changed or altered.

**New Benzothieno[3,2-*d*]-1,2,3-triazines with Antiproliferative Activity: Synthesis, Spectroscopic Studies, and Biology Activity**

Leave this area blank for abstract info.

Antonino Lauria,* Alessia Alfio, Riccardo Bonsignore, Carla Gentile, Annamaria Martorana, Giuseppe Gennaro, Giampaolo Barone, Alessio Terenzi, Anna Maria Almerico



Bioorganic & Medicinal Chemistry Letters
journal homepage: www.elsevier.com

# New Benzothieno[3,2-*d*]-1,2,3-triazines with Antiproliferative Activity: Synthesis, Spectroscopic Studies, and Biology Activity

Antonino Lauria,* Alessia Alfio, Riccardo Bonsignore, Carla Gentile, Annamaria Martorana, Giuseppe Gennaro, Giampaolo Barone, Alessio Terenzi, Anna Maria Almerico

*Dipartimento di Scienze e Tecnologie Biologiche, Chimiche e Farmaceutiche (STEBICEF), Università di Palermo - Via Archirafi, 32 - 90123 Palermo, Italy.*

ARTICLE INFO

*Article history:*
Received
Revised
Accepted
Available online

*Keywords:*
Benzothienotriazines
Antiproliferative Activity
Spectroscopic Studies
Cell-cycle analysis
VLAK

ABSTRACT

New benzothieno[3,2-*d*]-1,2,3-triazines, together with precursors triazenylbenzo[*b*]thiophenes, were designed, synthesized and screened as anticancer agents. The structural features of these compounds prompted us to investigate their DNA binding capability through UV-Vis absorption titrations, circular dichroism, and viscometry, pointing out the occurrence of groove-binding. The derivative 3-(4-methoxy-phenyl)benzothieno[3,2-*d*]-1,2,3-triazin-4(3*H*)-one showed the highest antiproliferative effect against HeLa cells and was also tested in cell cycle perturbation experiments. The obtained results assessed for the first time the anticancer activity of benzothieno[3,2-*d*]-1,2,3-triazine nucleus, and we related it to its DNA-binding properties.



Fused heterocyclic systems are frequently focus of interest in medicinal chemistry. Due to the presence of heteroatoms, these small molecules, generally aromatic, could mime endogenous substrates displaying a wide spectrum of biological effects. The 1,2,3-triazine scaffold represents a versatile lead structure with multitude of interesting applications in numerous pharmacological fields. Tubercidin, is a clear example of 1,2,3-triazine derivative with potent antimicrobial activity.[1] Fusion of heterocyclic nuclei like indole, thiophene and pyrazolo rings to the 1,2,3-triazine core generates series of derivatives with enhanced biological activities (e.g. antitumor,[2–4] antimicrobial,[5] antiviral,[6] analgesic properties[7]).

In this light, our research interests are focused on planning heterocyclic compounds endowed with anticancer activity.[8] Therefore, the synthesis of designed compounds leads to the screening of new small molecules able to inhibit cancer cell proliferation. Among planned structures, characterized by the presence of annelated triazine ring and extended aromaticity, derivatives of the nuclei indolo[1,2-*c*]-1,2,3-benzotriazine **1** and pyrrolo[2,1-*c*]-1,2,4-triazine **2** (Figure 1) displayed a good antitumor effect at micromolar concentration.[4,9] Recently, also heterocyclic compounds **3a,b**, both embedded with properly decorated triazine nucleus and sulfur atom in the skeleton, demonstrated excellent *in vitro* and *in vivo* antiproliferative activity.[10] In the same way, indolo[3,2-*e*]-1,2,3-triazolo[1,5-*a*]pyrimidine derivatives of type **3c**, designed as DNA interactive agents, have shown good antiproliferative activity against a wide panel of human tumor cell lines.[11]

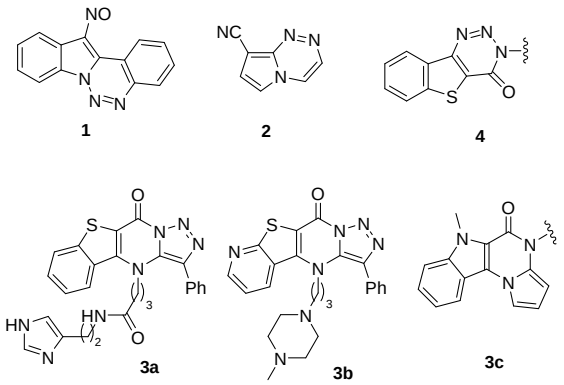


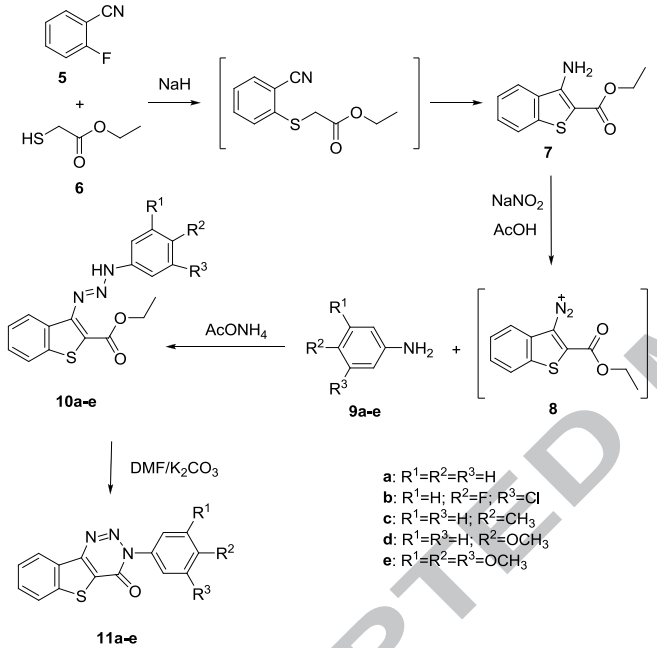

**Figure 1.** Designed heterocyclic compounds with wide spectra of antiproliferative activity.

On the basis of these data, we decided to investigate the benzothieno[3,2-*d*]-1,2,3-triazine core **4**, which presents three important requirements: the 1,2,3-triazine nucleus, the polyaromatic system, and the sulfur atom. This choice was driven by the opportunity of combining two different structural features present in several DNA anticancer drugs: the 1,2,3-triazine core and the aromaticity of polyheterocyclic moieties, enhanced by the presence of the isosteric sulfur atom. In particular, the 1,2,3-triazine ring may undergo ring-opening with the release of alkylating species, and the planar heterocyclic scaffold could electrostatically bind DNA.[12] Moreover, as below reported, the synthesis of the 1,2,3-triazine nucleus was achieved by cyclization of the corresponding triazene derivatives. In this regard, several triazene structural analogues showed antibacterial and antifungal effects,[13] in addition to an interesting antitumor

activity as DNA alkylating agents.[14] Indeed, even though few papers described the synthesis of the benzothieno[3,2-*d*]-1,2,3-triazine core,[15] only the anti-hyperlipidemic properties of few tetrahydro derivatives have been reported,[16–18] pointing out the lack of data as anticancer candidate drug.

In this paper we have explored a synthetic strategy leading for the first time to benzothieno[3,2-*d*]-1,2,3-triazine compounds **11**. DNA binding experiments and biological assays as antiproliferative agents against HeLa cells, on synthesized derivatives and their triazenes precursors, were carried out. Moreover, we have analysed the effect of the derivative with the highest antiproliferative activity on cell cycle. Finally, i*n silico* studies, through the chemometric protocol VLAK (Virtual Lock-and-Key),[19] were also performed.[20]

A well known synthetic route was applied to obtain the benzothieno[3,2-*d*]-1,2,3-triazine-4(3*H*)-one derivatives of type **11** (Scheme 1).[21,22]




**Scheme 1.** Synthesis of properly decorated benzothieno[3,2-*d*]-1,2,3-triazines **11a-e**.

The key intermediate ethyl 3-aminobenzo[*b*]thiophene-2-carboxylate **7** was obtained, in excellent yield, by reaction of 2-fluorobenzonitrile **5**, ethyl thioglycolate **6**, and sodium hydride, using dry dimethyl sulfoxide (DMSO) as reaction solvent (Scheme 1). Diazotization of **7** in acetic acid and consequent coupling of the resulting diazonium salt **8** with primary amines **9a-e**,[23,24] allowed the isolation of the ethyl 3-(3-phenyltriaz-1-enyl)benzo[*b*]thiophene-2-carboxylates **10a-e**. Finally, title benzothieno[3,2-*d*]-1,2,3-triazin-4(3*H*)-ones **11a-e** were achieved by intramolecular cyclization of triazenes **10**, heating the reaction mixture at 80 °C under basic condition.

The benzothieno[3,2-*d*]-1,2,3-triazine compounds of type **11** and their precursors **10** were tested to verify the DNA binding ability, through different techniques: UV-Vis absorption spectroscopy, circular dichroism (CD), and viscosity studies, using calf thymus DNA (ct-DNA). These methodologies were recently applied by us in the DNA binding studies of heterocyclic compounds and metal-complexes.[25] Compound **10d** was found to be slightly insoluble in buffered aqueous solutions at the DMSO/ethanol percentage used in all experiments, giving scattering during spectroscopic titration and therefore it was not further investigated. All tested compounds share absorption bands at about 250 and 280 nm; furthermore, a characteristic absorption band, in a region where DNA does not absorb is noticeable in **10a** (389 nm), **10b** (392 nm), **10c** (394 nm),**10e** (398 nm), **11a** (340 nm), **11b** (348 nm), **11c** (364 and 339 nm), **11d** (342 nm) and **11e** (333 nm). The linear trend of the band intensities ($A_{max}$) with the compounds molar concentration shows that the Lambert-Beer law is nicely fulfilled in the concentration ranges used for the interaction studies.

Among linear derivatives, only compounds **10a** and **10e** show modifications in their UV-Vis spectra in presence of increasing amounts of B-DNA. In detail, absorption band at 389 nm for **10a** shows hypochromism of about 12% whereas the one sited at 398 nm for **10e** displays hypochromism of about 11%. Furthermore, a red shift of about 10 nm and 4 nm is noticeable respectively for **10a** and **10e**.

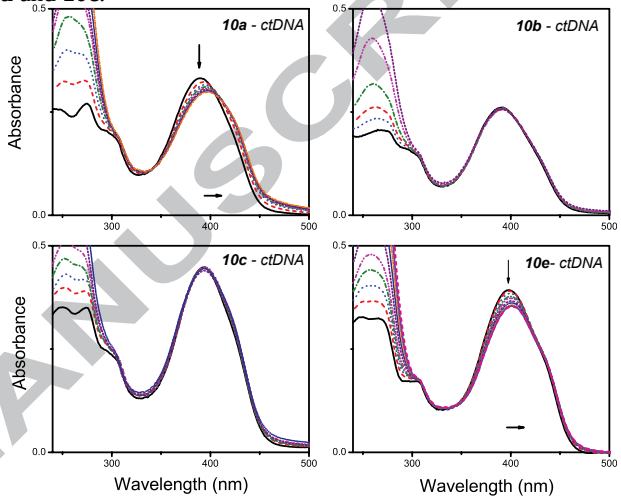



**Figure 2.** Absorption spectra of **10a-c** and **10e** in presence of increasing amounts of ct-DNA. [**10a**]=17.0 μM, [DNA]=0.0-85.0 μM. [**10b**]=14.6 μM, [DNA]=0.0-74.0 μM. [**10c**]=18.4 μM, [DNA]=0.0-48.3 μM. [**10e**]=18.5 μM, [DNA]=0.0-74.0 μM. The arrows indicate the change upon DNA addition.

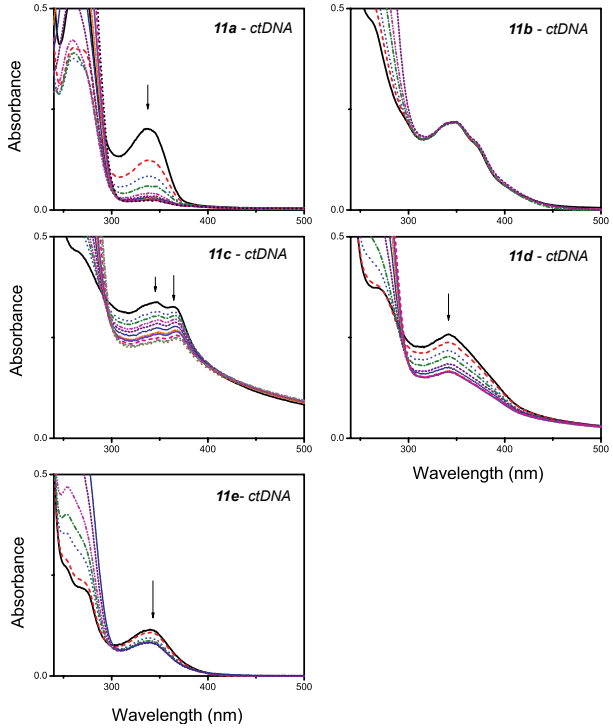



**Figure 3.** Absorption spectra of **11a-e** in presence of increasing amounts of ct-DNA. [**11a**]=27 μM, [DNA]=0.0-159.6 μM. [**11b**]=60 μM, [DNA]=0.0-302.75 μM. [**11c**]=51.4 μM, [DNA]=0.0-222.7 μM. [**11d**]=54 μM, [DNA]=0.0-149 μM. [**11e**]=29.1 μM, [DNA]=0.0-87.8 μM. The arrows indicate the change upon DNA addition.

In each titration of triazine derivatives (Figure 3), with the exception of **11b**, the characteristic spectrum is modified by the addition of increasing amounts of ct-DNA, showing a clear hypocromic effect.

In details, the absorption band of **11a** at 338 nm shows hypochromism of about 85%, **11c** at 339 nm shows hypochromism of about 25%; the absorption band of **11d** at 342 nm shows hypochromism of about 28%; the absorption band of **11e** at 333 nm shows hypochromism of about 29%.

On the other hand, the band at 346 nm of **11b** is not modified by the presence of increasing amounts of DNA, suggesting thus the absence of any perturbation of its electronic structure (Figure 3). Interestingly, the lack of interaction between **11b** and DNA might be due to the presence of Cl and F in the derivative, which increases the lipophilic properties of the molecule.

In order to obtain the DNA-binding constant ($K_b$) and stoichiometry (n) of the tested compounds, the results reported in Figure 2 and 3 have been analyzed by means of the McGhee-von Hippel equation (1):[26]

$$r/L = K_b(1-nr)\{(1-nr)/[1-(n-1)r]\}^{(n-1)} \qquad (1)$$

where $r$ is the ratio between the concentration of bound ligand and $DNA_{phosphate}$, $L$ is the concentration of free ligand, $K_b$ is the DNA-binding constant and $n$ is the number of $DNA_{phosphate}$ binding sites occupied by a bound ligand.

Results of data fitting are shown in Figure 4 and summarized in Table 1. As evident, linear derivatives **10a,e** interact with B-DNA with a stoichiometry that is almost two-fold than that one of cyclic derivatives **11a**, **11c-e**. Moreover, obtained binding constants are higher for linear than those of cyclic derivatives. This is in agreement with the statistical treatment of McGhee-von Hippel, by which the available DNA-binding sites, after ligand binding, are as less as much sites are bound, since each bound molecule hinders more than one free site.

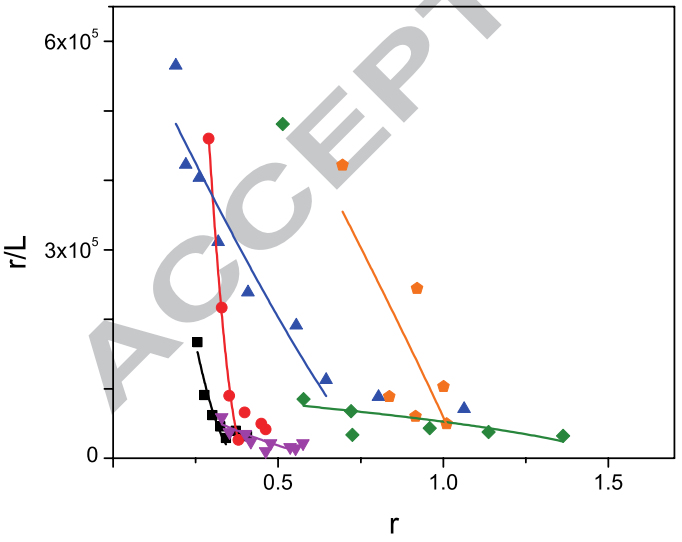



**Figure 4.** Scatchard plots of the absorption titrations shown in Figures 3: **10a**, black squares; **10e**, red circles; **11a**, blue up triangles; **11c**, purple down triangles; **11d**, green diamonds; **11e**, orange pentagons. Solid lines show the fits to McGhee-von Hippel equation (1). DNA-binding constants ($K_b$) and binding stoichiometries (n) are reported in Table 1.

**Table 1**. DNA-binding constant ($K_b$) and binding stoichiometry obtained using equation (1).

| Cpd | $K_b$ ($M^{-1}$) | n |
|---|---|---|
| **10a** | $9.87\times10^5$ | 2.53 |
| **10e** | $4.14\times10^6$ | 2.43 |
| **11a** | $6.67\times10^5$ | 1.23 |
| **11c** | $1.23\times10^5$ | 1.49 |
| **11d** | $9.72\times10^4$ | 0.66 |
| **11e** | $9.84\times10^5$ | 0.95 |

CD is a useful technique to monitor changes in the DNA structure following drug–DNA interactions, since its CD signals, characterized by a positive band centered at 275 nm and a negative band at 240 nm (black solid lines in Figure 5 and 6),[27] are sensitive to little variations of the DNA chiral conformation.[28]

For such a reason, we recorded CD titrations of DNA solutions with increasing amounts of binders. As shown in Figure 5 and Figure 6, results demonstrate that CD spectra of DNA never changes drastically as it would be if binders were acting as intercalators.[29,30] Only compounds **10a**, **11c** and **11d** induce slight changes in the CD spectrum of DNA, whereas nothing occurs with compounds **10e**, **11a** and **11e** (Figure 5 and 6).

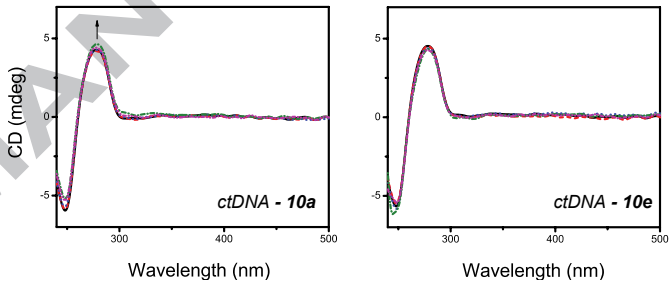



**Figure 5.** Circular dichroism spectra of ct-DNA (black line) in presence of increasing amounts of **10a** and **10e**. [DNA]=53 μM; [**10a**]=0.0-35.00 μM. [DNA]=53 μM, [**10e**]=0.0-35.00 μM. The arrows indicate the change upon binder addition. $R_1$ = [Binder]/[DNA].

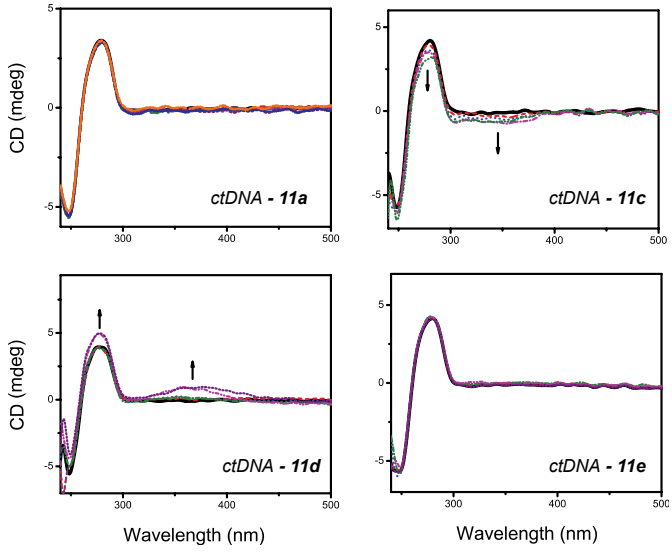



**Figure 6.** Circular dichroism spectra of ct-DNA (black line) in presence of increasing amounts of **10a** and **10c-e**. [DNA]=53 μM, [**11a**]=0.0-41.27 μM. [DNA]=53 μM, [**11c**]=0.0-24.96 μM. [DNA]=53 μM, [**11d**]=0.0-35.04 μM. [DNA]=53 μM, [**11e**]=0.0-35.04 μM. The arrows indicate the change upon binder addition. $R_1$ = [Binder]/[DNA].

In details, the DNA dichroic band at 275 nm decreases in the presence of **11c** and it increases at the higher molar ratios studied

for compounds **10a** and **11d**. Moreover CD induced bands (ICD) in the range 300-400 point out that **11c** and **11d** act as new CD chromophores after interacting with polynucleotide. These results allow us to argue that no deep conformational changes in the DNA helical structure occur due to interaction with the synthesized compounds, as it is referred in literature for typical groove binders.[31]

It is known that molecules able to intercalate DNA could induce an unwinding of the duplex supercoils and elongation of the DNA, giving rise to changes in its hydrodynamic properties, especially an increase of its solution viscosity.[32,33] In the absence of more detailed structural data (X-ray crystallography or NMR), viscosity experiments provide a critical test for intercalative binding.[34–36] Thus, viscosity measurements were carried out in order to definitively exclude the intercalation mechanism for our compounds.

As a matter of fact, the relative viscosity of a $5.3\times10^{-5}$ M DNA solution is not significantly affected by the presence of all the compounds investigated (see Figure S1 in supplementary material). Therefore, these results suggest the absence of DNA intercalation and stress the conclusion that compounds **10a**, **10e**, **11a**, **11c-e** act as groove binders. On the contrary, no interaction is observed for **10b**, **10c**, and **11b**.

The derivatives **11** and, due to the interesting antiproliferative activity showed by triazenes compounds in our previous findings,[37] the intermediate triazenes **10,** were tested using MTT based cell viability assay against HeLa cancer cell line. Dacarbazine (DTIC), an alkylating triazine employed in the treatment of various malignancies,[38] and its triazene precursor temozolomide (TMZ), used against malignant brain tumors,[39] were tested as internal reference compounds. All synthesized compounds showed concentration-dependent and time-dependent cytotoxicity, but the antiproliferative effect was achieved with diverse efficacy. The GI50 values of selected compounds tested at 24h and 48h are shown in Table 2. In general, triazenes **10** showed poor antiproliferative effects and displayed GI50 values > 20 µM at 24 h and > 10 µM at 48 h, whereas, in most cases, the corresponding triazines **11** were more active. This may indicate that the thiophene ring, unlike what is observed for pyrrole derivatives,[37] makes the triazene moiety more stable and consequently less active in biological environment.

**Table 2.** Cytotoxicity of triazines **11** and triazenes **10** expressed as GI50 values against HeLa cell line.

| Cpd | GI50±SE (µM) | | Cpd | GI50±SE (µM) | |
|---|---|---|---|---|---|
| | **24 h** | **48 h** | | **24 h** | **48 h** |
| **10a** | 49.34 ± 3.42 | 26.09 ± 2.23 | **11a** | 18.57± 1.52 | 9.79 ± 0.91 |
| **10b** | 31.36 ± 2.81 | 12.87 ± 0.89 | **11b** | 6.35 ± 0.74 | 2.45 ± 0.22 |
| **10c** | 42.08 ± 4.13 | 18.27 ± 2.37 | **11c** | 5.32 ± 0.47 | 1.88 ± 0.23 |
| **10d** | 49.09 ± 3.91 | 17.39 ± 1.17 | **11d** | 4.37 ± 0.83 | 1.24 ± 0.63 |
| **10e** | 22.76 ± 1.97 | 14.44 ± 1.05 | **11e** | 20.69 ±1.31 | 9.44 ± 0.73 |

Among triazenes, derivative **10e** is the most active, but no significant difference in GI50 values was observed between this compound and the corresponding triazine **11e**. Among triazines **11b**, **11c**, and **11d** showed very strong cytotoxic effect with GI50 at 24 h in the low micromolar range and at 48 h in the micromolar range. Finally, as determined by the LDH (lactate dehydrogenase) assay, all the tested compounds in the concentration range 1-50 µM did not affect the plasma membrane integrity of the HeLa cell line (data not shown). It is worth noting that synthetized triazines and their triazene precursors in our experimental setup result more potent antiproliferative agents, against HeLa cell line, then the drugs DTIC and TMZ. Indeed, when tested in a concentration range from 50 to 1 µM, neither DTIC nor its triazene precursor TMZ showed antiproliferative effects (GI50>50µM).

The derivative **11d,** which showed the highest antiproliferative effect, was also tested in cell cycle perturbation experiments to evaluate its potential influence on cell-cycle distribution. Flow cytometric analysis was performed after 12 h and 24 h of incubation in order to detect the shifts in cell cycle distribution before a significant amount of cells underwent apoptosis. The working concentrations of compound **11d** were fixed at 1x and 2x its GI50 value at 24 h.

The histograms in Figure 5 represent the percentage of cells in the respective cell cycle phase ($G_1$, S, and $G_2$/M), along with the percentage of cells in the sub-$G_1$ (dead cells) obtained by flow cytometry. Untreated HeLa cells showed a normal diploid distribution presenting fast proliferation characteristics, with S + $G_2$/M phase cells accounting for over 35% of the total cells. Strong suppression of the G2/M phase with greater accumulation of cells in the G0/G1 was observed after **11d** exposure. The effect is concentration-dependent and in our experimental conditions treatment with 2xGI50 (8.8 µM) induced cell cycle arrest as a result of 12 h treatment whereas after cell exposure to 1xGI50 (4.4 µM) cell cycle arrest was observed after 24 h treatment. Moreover, after 24 h treatment with compound **11d** at 2xGI50, a significant increase in the cell population in the sub-G1 phase was observed, which is indicative of apoptotic cells.

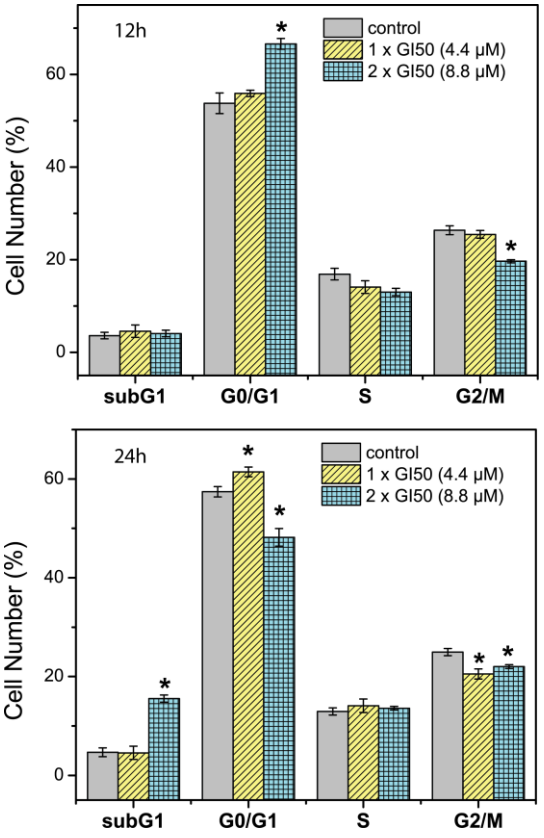



**Figure 5.** Effects of **11d** derivative at 2x and 1x of its GI50 value on the cell cycle distribution of HeLa cells following 12 h and 24 h treatment. Results are expressed as the mean of two independent experiments, performed in duplicate ±SE. Statistical analyses were performed using the Student's t test to determine the differences between the datasets. * denote significant differences ($p < 0.05$) from untreated cells (control)

The specific arrest of cells in the G1 phase suggests that cell exposure to **11d** can lead to perturbation, and consequent prevention, of DNA synthesis. Our results from spectroscopic studies suggest that DNA-binding ability of **11d** is correlated to capacity of this derivative to act as minor groove binder. DNA-interaction, even only reversible, can cause inhibition of DNA-dependent functions, including DNA-replication, by interference with the catalytic activity of important regulatory proteins, such as DNA-helicase and topoisomerase I and II.[40,41] Inhibition of DNA-Topoisomerase-I by groove ligands has already been shown and the ability of these DNA-binders to inhibit the enzyme was attributed to direct interactions with DNA at the minor groove.[42]

On the other hand, arrest of cells in the G1 phase is thought to be an important cellular defense mechanism that prevents replication of damaged DNA,[43] and we cannot exclude the possibility that cell exposure to **11d** may produce DNA-damage. As shown in literature, DNA damage can results also by non covalent-interactions after cell exposure to minor groove binders.[44,45]

Moreover, despite spectroscopic studies suggest no direct alkylating ability of **11d**, as is the case for classical minor groove ligands,[46] the possibility that in cellular environment **11d** may produce adducts with alkylating capacity, similarly to what reported for other minor groove binders, cannot be excluded.[47]

Recent *in silico* approaches for the design of heterocyclic compounds with biological activity[48,49] allowed us to study specific biological targets involved in carcinogenic processes,[50–52] as well as to improve the antiproliferative activity of different classes of heterocyclic compounds.[48,53–55]

The new benzothieno[3,2-*d*]-1,2,3-triazines, proposed herein, were submitted to the *in silico* protocol VLAK,[19] with the aim to get information on the expected mechanism of action.

The first step of the VLAK protocol is the conversion of the mechanism of action or the biological target in a "lock model" in which the keys (the structures) are "fitted".[19]

The NCI AntiCancer Agent Mechanism (ACAM) Database was chosen as source of biological data for the construction of the VLAK "lock models". It consists of a repository of structures with anticancer activity and a reasonably well-known mechanism of action. Drug screening data are available for each structure as measurement of their growth inhibition ability over a panel of about 60 human tumour cell lines, and all tested molecules are explicitly designed as a training set for neural network and multivariate analysis.[56,57]

In particular, this database is made up by 121 anticancer drugs classified according to their mechanism of action, which can be considered like the biological target in the protocol. The NCI ACAM Database was chosen because it was demonstrated that molecular descriptors, by means of multivariate analysis, are able to discriminate the drugs action mechanisms.[58]

The VLAK protocol, through a calculation procedure detailed in the supplementary material, assigned to benzothieno[3,2-*d*]-1,2,3-triazine compounds a percentage of affinity (A%) with respect to six mechanisms of action, including alkylating agents, antimitotic agents, topoisomerase I inhibitors, topoisomerase II inhibitors, RNA/DNA antimetabolites, and DNA antimetabolites (Table 3).

**Table 3.** VLAK results on benzothieno[3,2-*d*]-1,2,3-triazine derivatives

| | MA (A%)* | | | | | |
|---|---|---|---|---|---|---|
| Compound | **A** | **B** | **C** | **D** | **E** | **F** |
| **11a** | 2. 55 | 3. 44 | 4. 39 | 5. 48 | 6. 53 | 7. 53 |
| **11b** | 8. 58 | 9. 43 | 10. 38 | 11. 48 | 12. 53 | 13. 53 |
| **11c** | 14. 57 | 15. 46 | 16. 51 | 17. 49 | 18. 54 | 19. 54 |
| **11d** | 20. 59 | 21. 48 | 22. 53 | 23. 51 | 24. 56 | 25. 56 |
| **11e** | 26. 59 | 27. 49 | 28. 47 | 29. 54 | 30. 57 | 31. 55 |

***MA**, mechanism of action: **A%**, percentage of affinity; **A**, Alkylating Agents; **B**, Antimitotic Agents; **C**, Topoisomerase I Inhibitors; **D**, Topoisomerase II Inhibitors; **E**, RNA/DNA Antimetabolites; **F**, DNA Antimetabolites.

Benzothieno[3,2-*d*]-1,2,3-triazines of type **11** are well classified prevalently as alkylating agents (A). This trend, although not sharp, suggests that the antiproliferative activity of the benzothieno[3,2-*d*]-1,2,3-triazine derivatives could be related also to DNA interactions and consequent DNA damage and interference with DNA synthesis.

It is evident the spectroscopic studies give an account of the first step of the DNA interaction of the proposed compounds with a minor groove binding mode. Instead, the cell cycle analysis, performed on the most active compound **11d**, leads to hypothesize an antiproliferative mechanism which could involve covalent interactions, as in part confirmed by the *in silico* analysis VLAK. In fact, strong suppression of the G2/M phase with greater accumulation of cells in the G0/G1 was observed. The specific arrest of cells in the G1 phase suggests that cell exposure to **11d** can lead to consequences as dramatic as DNA damage with perturbation of DNA synthesis. Moreover, a significant increase in the cell population in the sub-G1 phase was observed, which is indicative of apoptotic cells. $G_1$ arrest can also be compatible with DNA-Topoisomerase-I inhibition and potential disturbances of replication or DNA damage.[59]

## Acknowledgments

This work was in part supported by FFR2012 - University of Palermo grant.

## References and notes

1. Kumar, R.; Singh, A.; Singh, J.; Singh, H.; Roy, R. K.; Chaudhary, A. *Mini-Rev. Med. Chem.* **2014**, *14*, 72.
2. Garuti, L.; Roberti, M.; Rossi, T.; Castelli, M.; Malagoli, M. *Eur. J. Med. Chem.* **1998**, *33*, 43.
3. Ali, M. M.; Mahmoud, A. E.; Rashad, A. E. *Asian J. Pharm. Clin. Res.* **2011**, *4*, 118.
4. Cirrincione, G.; Almerico, A. M.; Barraja, P.; Diana, P.; Lauria, A.; Passannanti, A.; Musiu, C.; Pani, A.; Murtas, P.; Minnei, C.; Marongiu, M. E.; La Colla, P. *J. Med. Chem.* **1999**, *42*, 2561.
5. Saravanan, J.; Mohan, S.; Roy, J. J. *Eur. J. Med. Chem.* **2010**, *45*, 4365.
6. Migawa, M. T.; Drach, J. C.; Townsend, L. B. *J. Med. Chem.* **2005**, *48*, 3840.
7. Viswanatha, G. L.; Akinapally, N.; Shylaja, H.; Nandakumar, K.; Srinath, R.; Janardhanan, S. *Maced. J. Med. Sci.* **2011**, *4*, 131.
8. Barone, G.; Terenzi, A.; Lauria, A.; Almerico, A. M.; Leal, J. M.; Busto, N.; García, B. *Coord. Chem. Rev.* **2013**, *257*, 2848.
9. Diana, P.; Barraja, P.; Lauria, A.; Montalbano, A.; Almerico, A. M.; Dattolo, G.; Cirrincione, G. *Eur. J. Med. Chem.* **2002**, *37*, 267.
10. Lauria, A.; Patella, C.; Abbate, I.; Martorana, A.; Almerico, A. M. *Eur. J. Med. Chem.* **2013**, *65*, 381.
11. Lauria, A.; Patella, C.; Dattolo, G.; Almerico, A. M. *J. Med. Chem.* **2008**, *51*, 2037.

12. Lauria, A.; Diana, P.; Barraja, P.; Almerico, A. M.; Cirrincione, G.; Dattolo, G. *J. Heterocycl. Chem.* **2000**, *37*, 747.
13. Nesterova, I. N.; Velezheva, V. S.; Alekseeva, L. M.; Padeiskaya, E. N.; Radkevich, T. P.; Baklanova, O. V.; Golovanova, E. A. *Pharm. Chem. J.* **1990**, *24*, 813.
14. Viviani, S.; Bonadonna, G.; Santoro, A.; Bonfante, V.; Zanini, M.; Devizzi, L.; Soncini, F.; Valagussa, P. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **1996**, *14*, 1421.
15. Beck, J. R.; Yahner, J. A. *J. Org. Chem.* **1976**, *41*, 1733.
16. Beck, J. R.; Yahner, J. A. *J. Org. Chem.* **1973**, *38*, 2450.
17. Viswanatha, G. L.; Janaki Priyadarshini, B.; Hanumanthappa, S.; Rangappa, S.; Janardhanan, S. *Oman Med. J.* **2012**, *27*, 388.
18. W. Schneller, S.; W. Clough, F. *Heterocycles* **1975**, *3*, 135.
19. Lauria, A.; Tutone, M.; Almerico, A. M. *Eur. J. Med. Chem.* **2011**, *46*, 4274.
20. Lauria, A.; Terenzi, A.; Martorana, A.; Gennaro, G.; Barone, G.; Almerico, A. M. *Lett. Drug Des. Discov.* **2014**, *11*, 15.
21. Neunhoeffer, H. *Chemistry of 1,2,3-triazines and 1,2,4-triazines, tetrazines, and pentazines*; The Chemistry of heterocyclic compounds□ ; v. 33; Wiley: New York,**1978**.
22. Chattaway, F. D.; Walker, A. J. *J. Chem. Soc. Resumed* **1927**, 323.
23. Day, B. F.; Campbell, T. W.; Coppinger, G. M. *J. Am. Chem. Soc.* **1951**, *73*, 4687.
24. Vaughan, K. *Can. J. Chem.* **1972**, *50*, 1775.
25. Lauria, A.; Bonsignore, R.; Terenzi, A.; Spinello, A.; Giannici, F.; Longo, A.; Almerico, A. M.; Barone, G. *Dalton Trans.* **2014**, *43*, 6108.
26. McGhee, J. D.; von Hippel, P. H. *J. Mol. Biol.* **1974**, *86*, 469.
27. Eriksson, M.; Nordén, B. In *Methods in Enzymology*; Jonathan B. Chaires, M. J. W., Ed.; Drug-Nucleic Acid Interactions; Academic Press, **2001**; Vol. Volume 340, pp. 68–98.
28. Waring, M. J. *J. Mol. Biol.* **1965**, *13*, 269.
29. Silvestri, A.; Barone, G.; Ruisi, G.; Anselmo, D.; Riela, S.; Liveri, V. T. *J. Inorg. Biochem.* **2007**, *101*, 841.
30. Barone, G.; Gambino, N.; Ruggirello, A.; Silvestri, A.; Terenzi, A.; Turco Liveri, V. *J. Inorg. Biochem.* **2009**, *103*, 731.
31. Terenzi, A.; Barone, G.; Piccionello, A. P.; Giorgi, G.; Guarcello, A.; Portanova, P.; Calvaruso, G.; Buscemi, S.; Vivona, N.; Pace, A. *Dalton Trans.* **2010**, *39*, 9140.
32. Cohen, G.; Eisenberg, H. *Biopolymers* **1969**, *8*, 45.
33. Reynisson, J.; Schuster, G. B.; Howerton, S. B.; Williams, L. D.; Barnett, R. N.; Cleveland, C. L.; Landman, U.; Harrit, N.; Chaires, J. B. *J. Am. Chem. Soc.* **2003**, *125*, 2072.
34. Lerman, L. S. *Proc. Natl. Acad. Sci. U. S. A.* **1963**, *49*, 94.
35. Dougherty, G.; Pilbrow, J. R. *Int. J. Biochem.* **1984**, *16*, 1179.
36. Satyanarayana, S.; Dabrowiak, J. C.; Chaires, J. B. *Biochemistry (Mosc.)* **1992**, *31*, 9319.
37. Diana, P.; Barraja, P.; Lauria, A.; Almerico, A. M.; Cirrincione, G.; Loi, A. G.; Musiu, C.; Pani, A.; La Colla, P.; Marongiu, M. E. *Eur. J. Med. Chem.* **1999**, *34*, 353.
38. Serrone, L.; Zeuli, M.; Sega, F. M.; Cognetti, F. *J. Exp. Clin. Cancer Res. CR* **2000**, *19*, 21.
39. Friedman, H. S.; Kerby, T.; Calvert, H. *Clin. Cancer Res.* **2000**, *6*, 2585.
40. Bachur, N. R.; Johnson, R.; Yu, F.; Hickey, R.; Applegren, N.; Malkas, L. *Mol. Pharmacol.* **1993**, *44*, 1064.
41. Drozdowska, D.; Rusak, M.; Miltyk, W.; Midura-Nowaczek, K. *Arch. Pharm. (Weinheim)* **2009**, *342*, 87.
42. Bugreev, D. V.; Vasyutina, E. L.; Ryabinin, V. A.; Sinyakov, A. N.; Buneva, V. N.; Nevinsky, G. A. *Antisense Nucleic Acid Drug Dev.* **2001**, *11*, 137.
43. Smith, M. L.; Fornace Jr., A. J. *Mutat. Res. Genet. Toxicol.* **1996**, *340*, 109.
44. Turner, P.; Denny, W. *Curr. Drug Targets* **2000**, *1*, 1.
45. Zihlif, M.; Catchpoole, D. R.; Stewart, B. W.; Wakelin, L. P. G. *Cancer Genomics - Proteomics* **2010**, *7*, 323.
46. Hurley, L.; Reynolds, V.; Swenson, D.; Petzold, G.; Scahill, T. *Science* **1984**, *226*, 843.
47. Fedier, A.; Fowst, C.; Tursi, J.; Geroni, C.; Haller, U.; Marchini, S.; Fink, D. *Br. J. Cancer* **2003**, *89*, 1559.
48. Lauria, A.; Abbate, I.; Gentile, C.; Angileri, F.; Martorana, A.; Almerico, A. M. *J. Med. Chem.* **2013**, *56*, 3424.
49. Lauria, A.; Tutone, M.; Barone, G.; Almerico, A. M. *Eur. J. Med. Chem.* **2014**, *75*, 106.
50. Lauria, A.; Ippolito, M.; Fazzari, M.; Tutone, M.; Di Blasi, F.; Mingoia, F.; Almerico, A. M. *J. Mol. Graph. Model.* **2010**, *29*, 72.
51. Lauria, A.; Ippolito, M.; Almerico, A. M. *Comput. Biol. Chem.* **2009**, *33*, 386.
52. Lauria, A.; Ippolito, M.; Almerico, A. M. *J. Mol. Graph. Model.* **2009**, *27*, 712.
53. Lauria, A.; Patella, C.; Abbate, I.; Martorana, A.; Almerico, A. M. *Eur. J. Med. Chem.* **2012**, *55*, 375.
54. Lauria, A.; Abbate, I.; Patella, C.; Martorana, A.; Dattolo, G.; Almerico, A. M. *Eur. J. Med. Chem.* **2013**, *62*, 416.
55. Mingoia, F.; Di Sano, C.; Di Blasi, F.; Fazzari, M.; Martorana, A.; Almerico, A. M.; Lauria, A. *Eur. J. Med. Chem.* **2013**, *64*, 345.
56. Weinstein, J. N.; Kohn, K. W.; Grever, M. R.; Viswanadhan, V. N.; Rubinstein, L. V.; Monks, A. P.; Scudiero, D. A.; Welch, L.; Koutsoukos, A. D.; Chiausa, A. J.; Et, A. *Science* **1992**, *258*, 447.
57. Van Osdol, W. W.; Myers, T. G.; Paull, K. D.; Kohn, K. W.; Weinstein, J. N. *JNCI J. Natl. Cancer Inst.* **1994**, *86*, 1853.
58. Lauria, A.; Ippolito, M.; Almerico, A. M. *QSAR Comb. Sci.* **2009**, *28*, 387.
59. Antony, S.; Agama, K. K.; Miao, Z.-H.; Hollingshead, M.; Holbeck, S. L.; Wright, M. H.; Varticovski, L.; Nagarajan, M.; Morrell, A.; Cushman, M.; Pommier, Y. *Mol. Pharmacol.* **2006**, *70*, 1109.


## Supplementary Material

Experimental methodologies and NMR spectra are available.