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Design, synthesis and in vitro anticancer evaluation of 4,6-diamino-1,3,5-triazine-2-carbohydrazides and -carboxamides

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ABSTRACT

Series of substituted 4,6-diamino-1,3,5-triazine-2-carbohydrazides and -carboxamides have been synthesised, based on molecular modelling of candidate structures related to the previously reported Rad6B-inhibitory diamino-triazinylmethyl benzoate anticancer agents TZ8 and TZ9. Synthesis of the target compounds was readily accomplished in two steps from aryl biguanides via reaction of phenylhydrazine or benzylamines with key 4-amino-6-(arylamino)-1,3,5-triazine-2-carboxylate intermediates. These new triazine derivatives were tested for in vitro anticancer activity against the Rad6B expressing human breast cancer cell lines MDA-MB-231 and MCF-7. Active compounds, such as the triazinyl-carbohydrazides **3a**–**e**, were found to exhibit low micromolar IC_{50} values particularly in the Rad6B-overexpressing MDA-MB-231 cell line.

The highly-regulated ubiquitin-proteasome system, responsible for degradation of >80% of cellular proteins, has proven to be a popular target for the development of new anticancer agents in recent years.[1] Research in this field has culminated in the clinical approval of bortezomib (Velcade®) as the first-in-class small molecule proteasome inhibitor for treatment of relapsed multiple myeloma and mantle cell lymphoma.[1] A major function of the protein ubiquitination system serves to polyubiquitinate (tag with small proteins) cellular proteins destined for proteasomal degradation.[2] However the role of ubiquitination in other important cellular functions such as cell signalling and DNA repair is now becoming more widely appreciated.[3] Three successive classes of enzymes of increasing structural and mechanistic class diversity mediate protein ubiquitination. The process starts with the ubiquitin-activating enzyme (E1) that forms a thioester bond between its active site cysteine and the carboxyl terminus of the ubiquitin protein. Transfer of ubiquitin to the family of thioester-linked E2 ubiquitin conjugating enzymes results in either direct transfer of ubiquitin to the protein substrate, or interaction with the larger family of E3 ubiquitin–protein ligases resulting in substrate mono- or poly-ubiquitination.

Extensive efforts in recent years to target various classes of E3 ubiquitin ligases deregulated in cancer cells has led to the development of a number of potential cancer therapeutics. Amongst the most well developed examples are the Mdm2 (E3 ligase)—p53 protein–protein interaction inhibitors known as the Nutlins, currently being studied in clinical trials for cancer.[4] A further example is provided by the disulfiram-based inhibitors of the E3 ligase enzyme BCA2 (breast cancer associated protein-2) discovered in our laboratories.[5]

Inhibitors of E2 ubiquitin conjugating enzymes are less well studied than their E3 counterparts, despite their potential as cancer drug targets in a number of cases. For example, the E2 enzyme Rad6B has been found to be essential for post-replication DNA repair, and Rad6B over-expression is reported in breast cancer cell lines and tumours. Constitutive Rad6B over-expression in non-transformed cells is associated with induction of cancer phenotypic changes including centrosome amplification, abnormal mitosis and aneuploidy.[6] The ability of Rad6B to ubiquitinate β-catenin leads to conjugates insensitive to proteasomal degradation. Consequent stabilisation and activation of oncogenic β-catenin provides further evidence of the therapeutic potential of Rad6B as a drug target, particularly in breast cancer.[7,8]

An important therapeutic advance in this area was the recent report from our laboratories of the first selective inhibitors of

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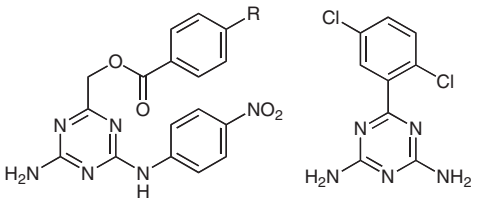

TZ8 (R=Me)
TZ9 (R=H)
(Rad6B-inhibitory anticancer)

Irsogladine
(anti-ulcer)

Figure 1. Biologically active tri-substituted 1,3,5-triazine derivatives.

Rad6B ubiquitin conjugating enzyme.[9] Virtual screening of a library of drug-like structures against a pharmacophore model generated from the conserved key residues stabilizing the E2-ubiquitin thioester intermediate, identified a substituted diamino-triazine core structure as a starting point for analogue synthesis. Triazine analogue synthesis coupled to in vitro anticancer evaluation in Rad6B-relevant models led to the identification of (4-amino-6-(arylamino)-1,3,5-triazin-2-yl)methyl 4-nitrobenzoates TZ8 and TZ9 (Fig. 1) as novel and selective Rad6B-inhibitory anticancer lead compounds.[9]

The tri-substituted 1,3,5-triazine scaffold plays an important role in medicinal chemistry, since a number of triazine based compounds are reported to possess useful biological properties. Biologically active tri-substituted triazines include the anti-gastric ulcer agent irsogladine (Fig. 1),[10] commonly used in Japan and also shown to possess anti-angiogenic/anti-metastatic activity;[11] plus other agents with anticancer,[12] antimalarial,[13] and antimicrobial[14a,b] properties. In this Letter, we detail the design, synthesis and in vitro anticancer evaluation of new 4-amino-6-(arylamino)-*N*-phenyl-1,3,5-triazine-2-carbohydrazides and related carboxamides, derived from our previous lead compounds TZ8 and TZ9.

A key limitation of the previously identified (4,6-diamino-1,3,5-triazin-2-yl)methyl benzoate derivatives TZ8 and TZ9 was their difficult and rather unreliable synthesis. Reaction of arylbiguanide and ethyl glycolate in particular proceeded in poor yield, accompanied by a number of by-products arising from the unprotected glycolate function.[9] We therefore studied the possibility of inverting the ester function to obtain compounds with similar promising anticancer activity via Rad6B inhibition using a molecular modelling approach to guide new compound design. New derivatives of TZ8 and TZ9 were studied by docking candidate compounds onto a published human Rad6B protein crystal structure (PDB ID: 2YB6).[15,16] For docking studies, we used the Rad6B active site template previously defined for triazines TZ8 and TZ9, based on minimum docking scores and binding energy orientations of test ligands.[9] Molecular docking interactions with structure 2YB6 were studied using MOE[17] and the LeadIt molecular docking software.[18] Compounds showing the lowest docking scores and binding energy interactions were selected for synthesis and anticancer evaluation. For example the triazine carbohydrazide derivative (**3c**) was incorporated deep inside the Rad6B binding pocket, making key interactions between the hydrazine nitrogen atoms and the Rad6B active site residues Cys88 and Asp90. Additional interactions between the anilino nitrogens of **3c** and Asn119/Gln93, and between the phenyl (hydrazide) ring and Leu89 were also apparent from our docking analysis (Fig. 2). The importance of these active site residues to the allosteric effect on Rad6B induced by E3 ligases, and the observation that no other E2 family members (with the exception of Rad6A) have residues corresponding to Gln93 or Asn119, suggest that these triazine carbohydrazides could be selective Rad6B inhibitors. In contrast our docking analysis suggested that the corresponding triazine carboxamides such as **6c** would be able to form hydrogen bonds with active site residues Cys88 and Asp90, but would fail to make additional interactions with other active site residues such as Leu89, Asn119 and Gln93 (see Supplementary information for additional docking interaction maps of triazines **3c** and **6c**).

The synthesis of the 4-amino-6-(arylamino)-*N*-phenyl-1,3,5-triazine-2-carbohydrazides (**3a**–**e**) was accomplished in two steps from arylbiguanide hydrochloride salts (**1a**–**e**), which were prepared from commercially available substituted aniline and dicyandiamide according to previously reported proedures.[9,12] Neutralisation of the arylbiguanide hydrochloride salt using sodium methoxide/methanol was followed by reaction with dimethyloxalate in refluxing methanol to give the intermediate methyl 4-amino-6-(arylamino)-1,3,5-triazine-2-carboxylates (**2a**–**e**) in 83–92% isolated yield following recrystallisation from methanol.[19] Reaction of intermediates (**2a**–**e**) with phenylhydrazine in refluxing ethanol, catalysed by glacial acetic acid, produced the target triazine carbohydrazides (**3a**–**e**)[20,21] in high yield (91–96%) following recrystallisation from methanol (Scheme 1).

A similar strategy was adopted for synthesis of new 4-amino-6-(arylamino)-*N*-benzyl-1,3,5-triazine-2-carboxamides (**6a**–**d**). In this case, arylbiguanides (**1a** and **b**) were cyclised with diethyloxalate in refluxing ethanol to generate the intermediate triazine

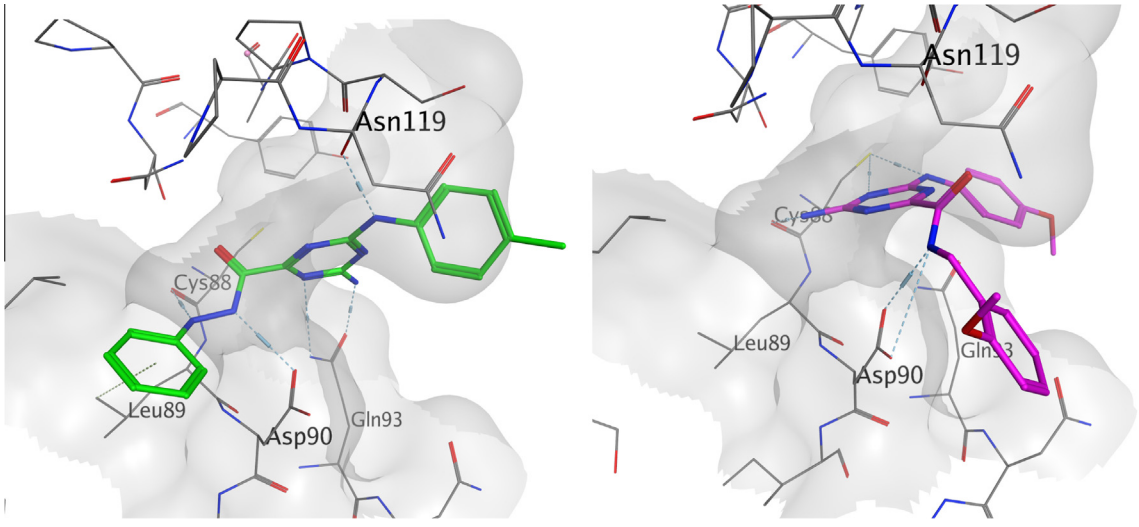

Figure 2. Docking interactions of triazine carbohydrazide **3c** and triazine carboxamide **6c** in the Rad6B active site.

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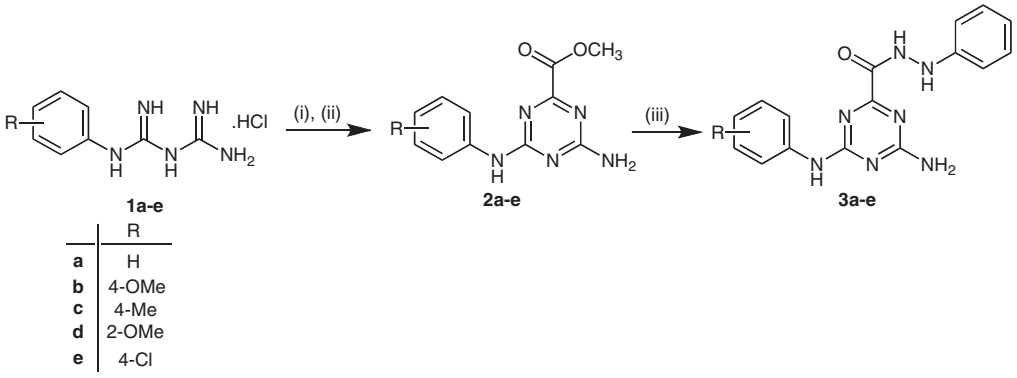
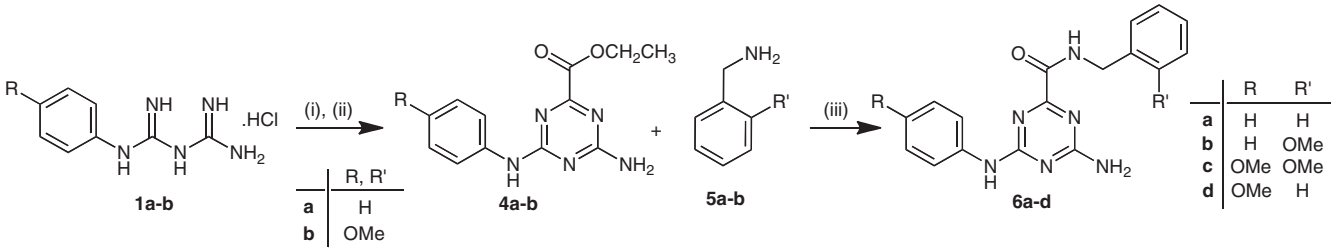


Scheme 1. Synthesis of 4-amino-6-(arylamino)-*N*-phenyl-1,3,5-triazine-2-carbohydrazides (**3a**–**e**). Reagents and conditions: (i) $NaOCH_3$, CH_3OH, rt, 3 h; (ii) dimethyloxalate, CH_3OH, reflux, 4 h; (iii) phenylhydrazine, AcOH, EtOH, reflux, 12 h.



Scheme 2. Synthesis of 4-amino-6-(arylamino)-*N*-benzyl-1,3,5-triazine-2-carboxamides (**6a**–**d**). Reagents and conditions: (i) $NaOCH_3$, CH_3OH, rt, 3 h; (ii) diethyloxalate, ethanol, reflux, 18 h; (iii) AcOH, dioxane, reflux, 18 h.

ethyl esters (**4a** and **b**) in 70–79% yield. Reaction of intermediates **4a** and **b** with benzylamines (**5a** and **b**) in dioxane under reflux, catalysed by acetic acid, produced the required triazine carboxamides (**6a**–**d**)[22,23] in good yield (62–74%) after recrystallisation from methanol (Scheme 2).

Evaluation of newly synthesized 4-amino-*N*-phenyl-6-(arylamino)-1,3,5-triazine-2-carbohydrazides (**3a**–**e**) and 4-amino-*N*-benzyl-6-(arylamino)-1,3,5-triazine-2-carboxamides (**6a**–**d**) was carried out in human breast cancer cell lines MDA-MB-231 and MCF-7 using the CellTiter-Blue® (CTB) assay to assess cell viability, as previously described (see Supplementary data).[24] The standard clinical anticancer drug doxorubicin and experimental proteasome inhibitor *N*-(benzyloxycarbonyl)-leucinylleucinylleucinal (MG-132)[25] were also tested for comparative purposes. MDA-MB-231 is an established metastatic Rad6B over-expressing breast cancer cell line, previously used in our studies on TZ8 and TZ9 analogues.[9] The established MCF-7 breast cancer cell line has also previously been used to study cellular effects of induced Rad6B.[26] Anti-proliferative data, expressed as mean values following testing on at least three separate occasions, are presented in Table 1. Activity of compounds in the nontransformed human breast epithelial cell line MCF10A was also studied using the CTB endpoint assay.

The results presented in Table 1 indicate structure–activity relationship trends. In general the Rad6B-expressing MDA-MB-231 cell line was the most sensitive to the effects of triazine hydrazide test compounds (**3a**–**e**) with IC_{50} values in the low micromolar range (2.48–4.79 μM) for compounds of this type. These values compare favourably with studies on TZ8 and TZ9 published previously,[9] although it should be noted that the values for the previously described triazines are compromised by poor solubility in tissue culture media. IC_{50} values for triazine hydrazides in the MCF-7 breast cancer cell line were found to be in the range between 17 and 53 μM. For the triazine carboxamides compounds **6a**, **6b** and **6d** were found to be essentially inactive in the MDA-MB-231 cell line; and in the case of **6a** and **6b** were also inactive in MCF-7 cells. In contrast 4-amino-*N*-(2-methoxybenzyl)-6-(2-methoxy-phenylamino)-1,3,5-triazine-2-carboxamide (**6c**) was found to be the most active compound tested against the MCF-7 cell line (IC_{50} 1.97 μM), with moderate activity against MDA-MB-231 (IC_{50} 32.9 μM). All newly tested compounds were less active than the anticancer drugs doxorubicin and MG-132. However it should be noted that these standard agents were found to exhibit inhibitory activity in the nontransformed breast epithelial cell line MCF10A (IC_{50} values of 1.11 and 0.29 μM for doxorubicin and MG-132, respectively), indicative of general toxicity. In contrast none of our newly synthesized test triazines achieved an IC_{50} value over the concentration range, giving IC_{50} values of >100 μM for the MCF10A cell line.

Table 1
Growth inhibitory activity (IC_{50}, μM) values in human breast cancer cell lines MDA-MB-231 and MCF-7, and the non-transformed epithelial cell line MCF10A, using the CTB assay (72 h incubation with test compound)

Compound	MDA-MB-231	MCF-7	MCF10A
3a	3.67 (0.46)	31.3 (3.1)	>100
3b	4.79 (0.40)	38.0 (2.5)	>100
3c	4.65 (0.13)	16.6 (0.2)	>100
3d	2.71 (0.21)	53.2 (4.8)	>100
3e	2.48 (0.72)	25.5 (5.2)	>100
6a	145.7 (8.5)	114.2 (6.2)	>100
6b	>100	101.2 (15.0)	>100
6c	32.9 (4.0)	1.97 (0.72)	>100
6d	>100	NT	NT
Doxorubicin .HCl	0.39 (0.19)	0.075 (0.006)	1.11 (0.14)
MG-132	0.18 (0.02)	0.13 (0.03)	0.29 (0.01)
TZ8	25[a]	NT	>100
TZ9	6[a]	NT	>100

Results are expressed as triplicate mean values (standard deviation in parentheses); NT = not tested.
[a] Approximate values due to low solubility in media.[9]

In summary, the computational design and synthesis of a series of 4-amino-6-(arylamino)-*N*-phenyl-1,3,5-triazine-2-carbohydrazides and 4-amino-6-(arylamino)-*N*-benzyl-1,3,5-triazine-2-carboxamides as potential anticancer agents targeting Rad6B has led to the discovery of a number of compounds with low micromolar IC_{50} activity specifically in Rad6B expressing human cancer cell lines. The triazine carbohydrazides (**3a**–**e**) were identified as the most active compounds of the series in MDA-MB-231 cells, and triazine carboxamide **6c** was the most active compound against MCF-7 cells. Our molecular modelling studies and cell line activity profile are consistent with Rad6B as a potential mechanistic target underpinning in vitro anticancer activity.

Acknowledgements

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Supplementary data

Supplementary data (Molecular modeling methodology and Rad6B interaction maps with triazines **3c** and **6c**; full compound characterisation data (mp, ^1H, ^{13}C NMR spectroscopy, mass spectrometry, % CHN analysis); cell culture protocols) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.09.087.

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19. *General method for synthesis of methyl 4-amino-6-(arylamino)-1,3,5-triazine-2-carboxylates.* A mixture of 1-arylbiguanide (**1a**–**e**, 60 mmol) and dimethyloxalate (7.0 g, 60 mmol) in methanol (100 mL) was stirred at room temperature under nitrogen for 2 h, and then heated under reflux for a further 4 h. The reaction mixture was allowed to cool, and the product crystals that formed were collected under vacuum and washed with a small quantity of cold methanol. Recrystallisation from methanol provided the product carboxylates (**2a**–**e**) in 83–92% yield which was used without further purification in the next step.
20. *General method for synthesis of triazine carbohydrazides (**3a**–**e**).* Phenylhydrazine (0.98 mL, 10 mmol) was added to a solution of the triazine ester intermediate (**2a**–**e**, 5 mmol) in ethanol (50 mL) containing glacial acetic acid (ca. 10 drops). The reaction mixture was heated under reflux for 12 h, then allowed and allowed to stand overnight. The resulting crystals were collected by vacuum filtration, dried and recrystallised from methanol to afford the triazine carbohydrazide products (**3a**–**e**) in 91–96% yield.
21. *Representative characterization data: 4-amino-N-phenyl-6-(tolylamino)-1,3,5-triazine-2-carbohydrazide (**3c**):* (95% yield). Mp 210–212 °C. ^1H NMR (DMSO-d_6) δ 2.27 (3H, s, CH_3), 6.75 (1H, t, *J* 7.8, H-4′), 6.80 (2H, d, *J* 8.0, H-2′, H-6′), 7.11 (2H, d, *J* 8.0, H-2, H-6), 7.18 (2H, t, *J* 7.8, H-3′, H-5′), 7.36 (2H, bs, NH_2), 7.69 (2H, d, *J* 8.0, H-3, H-5), 7.95 (1H, s, NH), 9.75 (1H, bs, NH), 10.06 (1H, s, NH). ^{13}C NMR (DMSO-d_6) δ 20.40 (CH_3), 112.24 (ArCH), 118.70 (ArCH), 120.22 (ArCH), 128.68 (ArCH), 128.85 (ArCH), 131.41 (ArC), 136.82 (ArC), 148.79 (ArC), 163.58 (ArC), 164.15 (ArC), 166.63 (ArC), 166.86 (ArC). MS (ESI^+) 336.2 (M^++1). Anal. Calcd for $C_{17}H_{17}N_7O$: C, 60.88; H, 5.11; N, 29.24. Found: C, 60.70; H, 4.91; N, 29.13.
22. *General method for synthesis of triazine carboxamides (**6a**–**c**).* A mixture of the triazine ester intermediate (**5a** and **b**, 2 mmol), (substituted)benzylamine (3 mmol), and glacial acetic acid (ca. 10 drops) in dioxane (30 mL) was heated under reflux for 18 h. The reaction mixture was allowed to cool then concentrated in vacuo and the residue poured onto ice. The resulting precipitate was collected by vacuum filtration, dried and recrystallised from methanol to afford the triazine carboxamide products (**6a**–**c**) in 62–74% yield.
23. *Representative characterisation data: 4-amino-N-(2-methoxybenzyl)-6-(4-methoxyphenylamino)-1,3,5-triazine-2-carboxamide (**6c**):* (74% yield). Mp 145–147 °C. ^1H NMR (DMSO-d_6) δ 3.74 (3H, s, OCH_3), 3.83 (3H, s, OCH_3), 4.42 (2H, d, *J* 6.1, CH_2N), 6.87 (2H, d, *J* 9.1, H-3, H-5), 6.93 (1H, t, *J* 8.2, H-5′), 7.03 (1H, d, *J* 8.1, H-3′), 7.22 (1H, t, *J* 6.3, H-4′), 7.29 (3H, m, H-6′+NH_2), 7.65 (2H, d, *J* 9.1, H-2, H-6), 8.51 (1H, t, *J* 7.6, CONH), 9.72 (1H, bs, NH). ^{13}C NMR (DMSO-d_6) δ 37.84 (CH_2N), 55.17 (OCH_3), 55.41 (OCH_3), 110.65 (ArCH), 113.66 (ArCH), 120.19 (ArCH), 121.79 (ArCH), 126.02 (ArC), 127.96 (ArCH), 128.40 (ArCH), 132.40 (ArC), 154.91 (ArC), 156.81 (ArC), 162.82 (ArC), 164.26 (ArC), 165.77 (ArC), 167.03 (ArC). MS (ESI^+) 381.2 (M^++1). Anal. Calcd for $C_{19}H_{20}N_6O_3$: C, 59.99; H, 5.30; N, 22.09. Found: C, 60.00; H, 5.17; N, 21.79.
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