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Original article

Synthesis of 2-aryl-4-(benzimidazol-2-yl)-1,2-dihydro[1,2,4]triazino-[4,5-*a*]benzimidazol-1-one derivatives with preferential cytotoxicity against carcinoma cell lines

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Abstract

In this paper we describe the preparation of some derivatives of 1,2,4-triazino[4,5-*a*]benzimidazol-1-ones (**5** and **6**), containing additional benzimidazole ring. These compounds were prepared using coupling reactions of diazonium salts with 1,1-bis(1-ethoxycarbonyl-benzimidazol-2-yl)methane (**2**) to obtain unstable hydrazones **4**, which readily undergo cyclization. Interestingly, the selected compounds demonstrated preferential cytotoxic activities against human carcinoma and glioma cell lines compared with leukemic cells. They showed significant activity against multidrug-resistant P-glycoprotein expressing cell lines but had less effect on multidrug-resistance protein 1 positive and topoisomerase IIα negative leukemias.

Keywords: Condensed 1,2,4-triazine; Benzimidazole; Hydrazones; Topoisomerase; MRP-1; Pgp

1. Introduction

Since a number of benzimidazole derivatives, including 2-aryl-4-(benzimidazol-2-yl)-1,2-dihydro[1,2,4]triazino-[4,5-a] benzimidazol-1-one [1], have proven biological activities – *inter alia* as peptide deformylase inhibitors [2,3], luteinizing hormone receptor antagonists [4], antitumor agents [5,6], antiulcerative agents [7,8], antiproliferative agents [9] and antimicrobial agents [10] – we have re-examined cyclization reactions that give rise to derivatives of 1,2,4-triazino[4,5-*a*]benzimidazole. Our current interest in compounds of this type increased after a series of the derivatives of the isosteric condensed 1,2,4-triazino[4,3-*a*]benzimidazole system which proved to be effective as selective aldose reductase inhibitors [11–13]. Recently, several lines of evidence demonstrated that aldose reductases are directly involved in cancerogenesis and tumor progression [14–16]. For this reason we have decided to test anticancer activity of those compounds under in vitro conditions using definitive endpoint cytotoxic assay.

2. Chemistry

The most efficient syntheses of 2-arylderivatives of 1,2,4-triazine include the cyclization reaction of hydrazones, which can be achieved via coupling reactions of diazonium salts with selected compounds with a reactive CH_2 group. The most common and proven syntheses of this type include the cyclization of ethyl arylhydrazono-cyanoacetyl carbamates resulting in 1-aryl-6-azauracils [17–19] and the analogous cyclization of arylhydrazones of ethoxycarbonylated amidines of mesoxalate acid generating 1-aryl-6-azacytosines [20]. Hydrazones of the last type include the arylhydrazones that can be generated by coupling diazonium salts with 2-(1-ethoxycarbonylbenzimidazole-2-yl)acetonitrile. However, the arylhydrazones are barely detectable owing to their very rapid cyclization [21–24].

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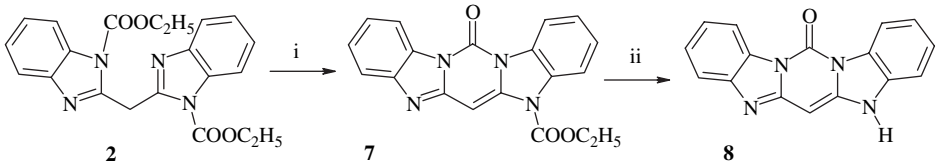


Scheme 1. Hydrolysis of diester **2** to compound **8**. *Reagents and conditions*: (i) water and pyridine, reflux 1 h; (ii) aqueous ethanolic HCl, reflux 1 h.

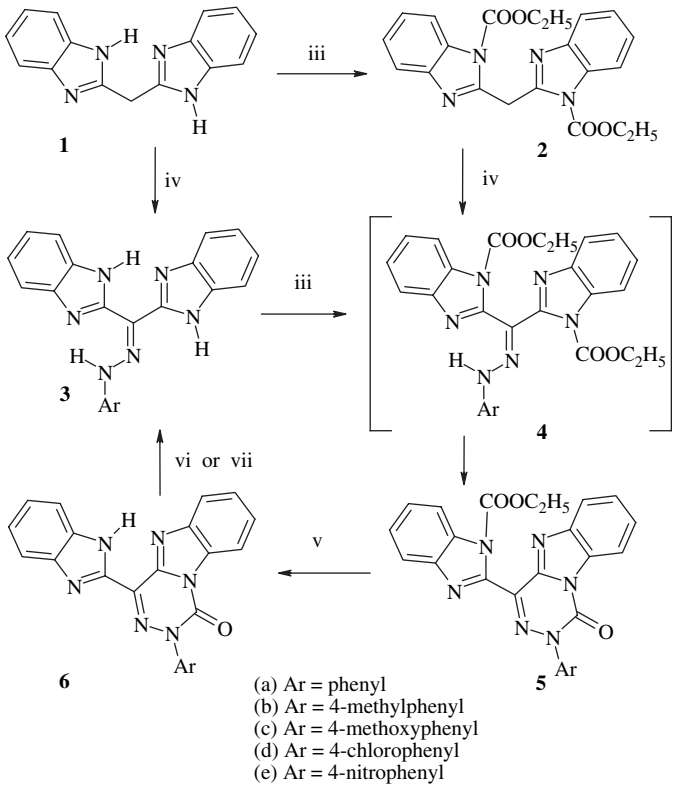
In attempts to prepare derivatives of 1,2,4-triazino[4,5-*a*]benzimidazole containing an additional benzimidazole ring, we used bis(benzimidazol-2-yl)methane (**1**) [25] as our starting material, and following its acylation using ethyl chloroformate in a pyridine medium we obtained the double ethoxycarbonylated derivative (**2**). It is very interesting to note that all our attempts to generate mono-ethoxycarbonyl products were unsuccessful. Even with the gradual addition of 1 eq of ethyl chloroformate to compound **1**, we could not obtain a mono-ethoxycarbonylated product, but only a mixture of the double ethoxycarbonylated derivative **2** and the unreacted starting material **1**. The mono-ethoxycarbonylated product was not obtained even from selective hydrolysis of compound **2** using 1 mol of NaOH; a mixture of several components was always obtained. We discovered that under more moderate conditions, boiling in aqueous pyridine, one of the ester groups was hydrolyzed, and in addition the pyrimidine ring formed, giving rise to ester **7**. Following another hydrolysis of compound **7** a condensed heterocyclic system, compound **8** is generated; this compound was previously produced via the reaction of bis(benzimidazol-2-yl)methane with phosgene [27]. It is very probable, based on ^{1}H NMR spectroscopic data that this compound is present in its 5*H*-tautomeric form rather than the 6*H*-tautomeric form as indicated earlier [27] (Scheme 1).

The most important point for further synthetic steps was to ensure the reactivity of the CH_2 group in compounds **1** and **2**. We discovered that the coupling reaction of diazonium salts with these components was very rapid. The diazotization of aniline and its derivatives and the diazonium salts with the CH_2 group of compound **1** derived from the coupling reaction of pyridine generate the corresponding hydrazones in good yields (**3a–3e**). However, during the analogous reaction of the diazonium salts with component **2**, hydrazones could not be successfully isolated (**4a–4e**), as they cyclized immediately to the corresponding 4-(1-ethoxycarbonylbenzimidazol-2-yl)-2-aryl-1,2-dihydro[1,2,4]triazino[4,5-*a*]benzimidazol-1-ones (**5a–5e**). This reaction was expected because of the previous experimental results [6–9]. Hydrazones **4a–4e** could not be separated even during the ethoxycarbonylation of hydrazones **3a–3e**. The reaction of hydrazones (**3a–3e**) with ethyl chloroformate in pyridine also gave rise to the abovementioned derivatives of 1,2,4-triazino[4,5-*a*]benzimidazole (**5a–5e**) (Scheme 2).

From the perspective of potential biological activity, we were mainly interested in compounds with a free NH group (**6a–6e**), which have structures that may allow them to intercalate with DNA double helices or interact with nucleic acids through intermolecular hydrogen bonding. Based on previous knowledge of the stability of 1,2,4-triazine ring system with respect to hydrolytic cleavage [26], we obtained compounds **6a–6d** without any difficulty following a short period of acid hydrolysis of compounds **5a–5e**, without any degradation of 1,2,4-triazine ring system. During long-term refluxing of compounds **5** and **6** with HCl or in aqueous pyridine, cleavage of the 1,2,4-triazine ring system occurred, giving rise to hydrazones **3a–3e**. In the case of NaOH, the hydrolysis and cleavage is very fast and compounds **6** are produced from compounds **5** after just 1 h of refluxing.

3. Biological experiments: results and discussion

The biological activities of the generated compounds of interest were examined by comparing their cytoxicity, using



Scheme 2. General scheme leading to 2-aryl-4-(benzimidazol-2-yl)-1,2-dihydro[1,2,4]triazino[4,5-*a*]benzimidazol-1-ones. *Reagents and conditions*: (iii) $ClCOOC_2H_5$ and pyridine; (iv) arene diazonium salt and pyridine; (v) aqueous ethanolic HCl, reflux 30 min; (vi) aqueous ethanolic HCl, reflux 48 h; (vii) water and pyridine, reflux 24 h.

the MTT assay (Table 1) [28,29], towards the drug-sensitive leukemia cell lines CEM (T-lymphoblastic) and K562 (myeloid), and the drug-resistant lines CEM-DNR-bulk (daunorubicin-resistant, topoisomerase IIα negative, multidrug-resistance protein 1 [MRP-1] positive and P-glycoprotein [Pgp] negative) and K562-Tax (topoisomerase IIα positive, MRP-1 negative and Pgp positive) [28]. The effects of the compounds were also analyzed (in parallel) on model cell lines of human lung adenocarcinoma (A549), colorectal carcinoma (HT-29, HCT116 p53wt, HCT116 p53mut), breast cancer (MCF-7), prostate cancer (PC-3) and glioblastoma (U118Mg). The compounds showed preferential activity against solid tumors compared to leukemia cells, which is unusual due to the intrinsic chemosensitivity of hematopoietic malignant diseases. Interestingly, biological activity decreased with the level of substitution at position 4. While compounds with the lipophilic substituents, 4-phenyl, 4-methylphenyl and 4-methoxyphenyl in compounds **3a–3c**, **5a–5c**, **6a–6c**, were highly active in leukemia cell lines at low micromolar concentrations, substitutions with 4-chlorophenyl or 4-nitrophenyl groups substantially decreased the cytotoxic potency (**3d–3e**, **5d–5e**, **6d–6e**). However, this was not the case in human carcinoma cell lines, where a higher level of substitution in position 4 demonstrated the opposite effect and generally increased the cytotoxic activity. Interestingly, higher activity was also observed in HCT116 cells expressing a mutated p53 oncosuppressor. Moreover, majority of the derivatives showed 2- to 10-fold decrease of cytotoxic activity against MRP-1 positive and topoisomerase IIα negative CEM-DNR-bulk cells, suggesting that the molecules are transported via MRP-1 and/or target the topoisomerase IIα gene. The decrease is considered as significant since it corresponds to reduction of cytotoxic activity in drug-sensitive versus resistant cell lines or primary tumor cells using classical anticancer agents [28,29]. However, those observations will require more detailed confirmatory studies.

4. Conclusions

We have successfully prepared derivatives of 1,2,4-triazino[4,5-*a*]benzimidazol-1-ones (**5** and **6**), containing an additional benzimidazole ring. The compounds were prepared using coupling reactions of diazonium salts with 1,1-bis(1-ethoxycarbonylbenzimidazol-2-yl)methane (**2**) to obtain unstable hydrazones **4**, which readily undergo cyclization. Analysis of their biological activity demonstrated that selected compounds possess preferential cytotoxic activities against human carcinoma cell lines and showed significant activity in the multidrug-resistant, Pgp expressing cell line K562-Tax, but were inactive in MRP-1 positive and topoisomerase IIα negative CEM-DNR-bulk cells. Structure–activity relationship showed the cytotoxic activity to be dependent on the substituent at position 4.

5. Experimental

5.1. Chemical synthesis

Melting points were determined on a Boetius stage and are uncorrected. The IR spectra were recorded in KBr wafers on an ATI Unicam Genesis FTIR instrument. The NMR spectra were registered on a Bruker Avance 300 MHz DRX spectrometer; chemical shifts are reported in parts per million, and the coupling constants *J* in hertz. Elemental analyses were performed with an EA 1108 Elemental Analyser (Fison Instruments). Mass spectrometric experiments were performed using an LCQ ion trap mass spectrometer (Finnigan MAT, San Jose, CA, USA).

5.1.1. 1,1-Bis(1-ethoxycarbonylbenzimidazol-2-yl)-methane (2)

Bis(benzimidazol-2-yl)methane (**1**) (10 g; 40.3 mmol) was dissolved in anhydrous pyridine (500 ml) on boiling and cooled to 5 °C. To this solution, ethyl chloroformate (9.3 ml; 97.3 mmol) was added dropwise with stirring. After 1 h of

Table 1
Cytotoxic activity of synthesized compounds on human malignant cell lines with varying tissue origins, drug resistance profiles and p53 gene status

Compound	Cytotoxicity (IC_{50}, μM)										
	CEM	CEM-DNR-bulk	K562	K562-Tax	A549	MCF-7	PC-3	HT-29	HCT116 p53wt	HCT116 p53mut	U118Mg
3a	13	27.9	114.5	143.8	3.8	83.2	248.3	81.9	162.1	17.6	160
3b	26.5	87.6	159.6	238.6	0.6	95.8	250	95.9	194.9	10.5	250
3c	10.9	96.7	116.5	235.4	0.5	215.1	250	114.4	35.7	66.5	250
3d	42.5	112.1	199.5	159.2	2.8	140.4	250	113.6	201	16.8	250
3e	58.4	140.3	190.3	100.9	0.7	94.8	218.4	17.7	11.7	4.7	250
5a	58.6	18.4	69.4	42.2	18	35.1	37.5	15.2	11.4	10.8	17.7
5b	25.5	29.6	139.5	53.7	71	134	165.1	96.2	46.3	44.4	147.3
5c	31.8	110.9	14	7.8	44.4	51.6	145.3	38.7	42.8	46.3	54.3
5d	31.8	110.9	14.1	7.9	4	21.3	149.8	29.7	7.7	6.4	12.4
5e	154.7	223.7	165.5	196.4	4.4	144	124.9	21.9	70.7	5.4	6.2
6a	12.2	45.6	23.5	42.7	11.5	74.8	13.6	8	38.1	8.6	12
6b	18.5	61.2	18.3	20.9	14.7	34.1	40.8	15.2	7.8	7.6	17.9
6c	3.5	35.4	8.7	7.5	8.7	10.7	24.6	6	6.9	3.6	10
6d	83.6	51.2	133.9	166.6	1.2	140.5	242.5	88.6	73.9	2.3	247
6e	102.9	190.2	209.5	250	2.2	250	250	248	250	250	250

Data represent mean values from 3–5 independent experiments with typical standard deviations within 10–15% of the average.

stirring, the reaction mixture was left to stand at 2 °C for 18 h. Then, it was diluted slowly with water under stirring to a total volume of 1500 ml. The precipitated solid was collected on a filter, washed with water, crystallized from ethanol (250 ml) and dried at 110 °C for 1.5 h. Yield 11.6 g (73.4%, colorless crystals), mp 158–160 °C. ^{1}H NMR (DMSO-d_6): δ 1.28 (t, 6H, CH_3), 4.42 (q, 4H, CH_2), 5.04 (s, 2H, $-CH_2-$), 7.32–7.43 (m, 4H, arom), 7.63 (d, 2H, arom, $J = 6.9$ Hz), 7.98 (d, 2H, $J = 6.9$ Hz); ^{13}C NMR (DMSO-d_6): δ 13.6, 33.9, 64.0, 114.7, 119.3, 124.2, 124.6, 132.5, 141.6, 149.6, 151.5; IR (cm^{-1}): 2992, 1742, 1548, 1454, 1377, 1337, 1189, 766; MS (ESI, *m/z*): 393.1 $[M + H]^+$. Anal. Calcd. for $C_{21}H_{20}N_4O_4$ (392.4): C 64.28, H 5.14, N 14.28. Found: C 64.33, H 5.03, N 14.14.

5.1.2. 1-Arylhydrazono-1,1-bis(benzimidazol-2-yl)methanes (**3a–3e**): general procedure

A solution of the corresponding aromatic amine (2.00 mmol) in a mixture of ice water (5 ml) and HCl (35%; 1.5 ml) was diazotized by sodium nitrite (138 mg; 2.00 mmol) in ice water (4 ml). The mixture was stirred in an ice bath for 15 min and then added dropwise to the solution of compound **1** (500 mg; 2.01 mmol) in pyridine (80 ml), which was boiled and then cooled to 2–5 °C. The mixture was left to stand at 0–5 °C for 24 h and then slowly diluted with water to a total volume of 300 ml. The precipitated yellow-orange solid was collected by suction, washed with water and dried. The sample for analysis was obtained by crystallization from a DMSO–ethanol mixture. The purity of these compounds was determined by TLC on silica gel plates using toluene as the mobile phase.

5.1.2.1. 1-Phenylhydrazono-1,1-bis(benzimidazol-2-yl)methane (**3a**).

Yield 92.3%, yellow solid, mp 293–295 °C dec. ^{1}H NMR (DMSO-d_6): δ 7.07 (t, 1H, arom, $J = 8.1$ Hz), 7.30 (t, 2H, arom, $J = 7.8$ Hz), 7.37 (t, 2H, arom, $J = 8.1$ Hz), 7.45 (t, 2H, arom, $J = 7.8$ Hz), 7.62 (d, 1H, arom, $J = 7.5$ Hz), 7.71 (d, 2H, arom, $J = 8.1$ Hz), 7.84 (d, 1H, arom, $J = 7.2$ Hz), 7.90 (d, 1H, arom, $J = 7.8$ Hz), 7.93 (d, 1H, arom, $J = 7.5$ Hz), 12.70 (bs, 1H, NH), 13.31 (bs, 1H, NH), 14.61 (s, 1H, NH); ^{13}C NMR (DMSO-d_6): δ 111.4, 113.2, 114.5, 118.3, 118.8, 118.9, 121.7, 122.5, 122.7, 123.2, 124.2, 129.5, 131.9, 134.2, 141.6, 143.0, 143.2, 146.0, 149.9. IR [cm^{-1}]: 3456, 3220, 1598, 1537, 1492, 1262, 750. MS (ESI, *m/z*) 353.2 $[M + H]^+$. Anal. Calcd. for $C_{21}H_{16}N_6$ (352.4): C 71.58, H 4.58, N 23.85. Found: C 71.49, H 4.55, N 23.76.

5.1.2.2. 1-(4-Methylphenylhydrazono)-1,1-bis(benzimidazol-2-yl)methane (**3b**).

Yield 85.9%, yellow-orange solid, mp 297–299 °C dec. ^{1}H NMR (DMSO-d_6): δ 2.33 (s, 3H, CH_3), 7.26 (d, 2H, arom, $J = 8.4$ Hz), 7.29–7.31 (m, 2H, arom), 7.36 (t, 2H, arom, $J = 7.8$ Hz), 7.62 (d, 2H, arom, $J = 8.4$ Hz), 7.82 (d, 2H, arom, $J = 7.2$ Hz), 7.87–7.95 (m, 2H, arom), 12.70 (bs, 1H, NH), 13.27 (bs, 1H, NH), 14.58 (s, 1H, NH). IR [cm^{-1}]: 3462, 3219, 1555, 1537, 1505, 1277, 735. MS (ESI, *m/z*) 367.2 $[M + H]^+$. Anal. Calcd. for $C_{22}H_{18}N_6$ (366.4): C 72.11, H 4.95, N 22.93. Found: C 71.97, H 4.83, N 22.89.

5.1.2.3. 1-(4-Methoxyphenylhydrazono)-1,1-bis(benzimidazol-2-yl)methane (**3c**).

Yield 89.3%, yellow-orange solid, mp 291–293 °C dec. ^{1}H NMR (DMSO-d_6): δ 3.78 (s, 3H, OCH_3), 7.03 (d, 2H, arom, $J = 9.0$ Hz), 7.20–7.39 (m, 4H, arom), 7.59 (d, 1H, arom, $J = 7.5$ Hz), 7.67 (d, 2H, arom, $J = 9.0$ Hz), 7.81 (d, 1H, arom, $J = 7.2$ Hz), 7.86–7.94 (m, 2H, arom), 12.68 (bs, 1H, NH), 13.24 (bs, 1H, NH), 14.57 (s, 1H, NH). IR [cm^{-1}]: 3455, 3223, 1562, 1501, 1233, 737. MS (ESI, *m/z*) 383.2 $[M + H]^+$. Anal. Calcd. for $C_{22}H_{18}N_6O$ (382.4): C 69.10, H 4.74, N 21.98. Found: C 68.99, H 4.62, N 22.09.

5.1.2.4. 1-(4-Chlorophenylhydrazono)-1,1-bis(benzimidazol-2-yl)methane (**3d**).

Yield 94.4%, yellow solid, mp 302–304 °C dec. ^{1}H NMR (DMSO-d_6): δ 7.28–7.39 (m, 4H, arom), 7.48 (d, 2H, arom, $J = 8.7$ Hz), 7.62 (bs, 1H, arom), 7.75 (d, 2H, arom, $J = 8.7$ Hz), 7.81 (d, 1H, arom, $J = 7.5$ Hz), 7.86–7.98 (m, 2H, arom), 12.80 (bs, 1H, NH), 13.28 (bs, 1H, NH), 14.66 (s, 1H, NH). IR [cm^{-1}]: 3452, 3224, 1538, 1485, 1261, 821. MS (ESI, *m/z*) 387.2 $[M + H]^+$. Anal. Calcd. for $C_{21}H_{15}ClN_6$ (386.9): C 65.20, H 3.91, N 21.72. Found: C 65.18, H 4.08, N 21.81.

5.1.2.5. 1-(4-Nitrophenylhydrazono)-1,1-bis(benzimidazol-2-yl)methane (**3e**).

Yield 96.7%, orange solid, mp over 360 °C dec. ^{1}H NMR (DMSO-d_6): δ 7.33 (t, 2H, arom, $J = 8.1$ Hz), 7.41 (t, 2H, arom, $J = 8.1$ Hz), 7.65 (d, 1H, arom, $J = 7.5$ Hz), 7.90 (d, 2H, arom, $J = 9.0$ Hz), 7.93–8.00 (m, 3H, arom), 8.31 (d, 2H, arom, $J = 9.0$ Hz), 13.00 (bs, 1H, NH), 13.38 (bs, 1H, NH), 15.00 (s, 1H, NH). IR [cm^{-1}]: 3420, 3223, 1596, 1562, 1504, 1333, 1265, 1109, 768. MS (ESI, *m/z*) 398.2 $[M + H]^+$. Anal. Calcd. for $C_{21}H_{15}N_7O_2$ (397.4): C 63.47, H 3.80, N 24.67. Found: C 63.40, H 3.74, N 24.56.

5.1.3. 2-Aryl-4-(1-ethoxycarbonylbenzimidazol-2-yl)-1,2-dihydro[1,2,4]triazino[4,5-a]benzimidazol-1-ones (**5a–5e**): general procedure

Method A: A solution of the corresponding aromatic amine (2.00 mmol) in a mixture of ice water (5 ml) and HCl (35%, 1.5 ml) was diazotized by sodium nitrite (138 mg; 2.00 mmol) in ice water (4 ml). The mixture was stirred in an ice bath for 15 min and then added dropwise to the solution of compound **2** (784.8 mg; 2.00 mmol) in pyridine (45 ml), which was cooled to 2–5 °C. The mixture was left to stand at 0–5 °C for 48 h and then slowly diluted with water to a total volume of 350 ml. The next day, the precipitated solid was collected by suction, washed with water and dried. The sample for analysis was obtained by crystallization from ethanol (for compound **5a**) ev. ethanol–chloroform mixture. The purity of these compounds was determined by TLC on silica gel plates using chloroform–methanol mixture (80: 1 v/v) as the mobile phase.

Method B: To a cooled solution (2–5 °C) of the corresponding compound **2** (0.5 mmol) in anhydrous pyridine (20 ml), ethyl chloroformate (0.12 ml; 1.25 mmol) was added

dropwise with stirring at 2–5 °C. After 1 h of stirring, the mixture was left to stand at 2 °C for 48 h. Then, it was diluted slowly with water under stirring to a total volume of 200 ml. The compound starts to precipitate after standing for some hours. The precipitated solid was collected on a filter, washed with water and crystallized.

*5.1.3.1. 2-Phenyl-4-(1-ethoxycarbonylbenzimidazol-2-yl)-1,2-dihydro[1,2,4]triazino[4,5-a]benzimidazol-1-one (**5a**).* Yield 80.2% (method A), 74.2 (method B), beige crystals, mp 187–189 °C. ^{1}H NMR (DMSO-d_6): δ 0.99 (t, 3H, CH_3), 4.27 (q, 2H, CH_2), 7.50–7.68 (m, 7H, arom), 7.77 (d, 2H, arom, $J = 7.8$ Hz), 7.92 (d, 1H, arom, $J = 7.8$ Hz), 7.97 (t, 1H, arom, $J = 4.2$ Hz), 8.11 (d, 1H, arom, $J = 8.1$ Hz), 8.43 (t, 1H, arom, $J = 4.2$ Hz); ^{13}C NMR (DMSO-d_6): δ 13.3, 64.6, 114.6, 115.0, 120.6, 120.7, 125.1, 126.0, 126.2, 126.6, 126.8, 128.5, 128.9, 129.3, 132.3, 133.6, 139.8, 141.5, 141.9, 143.3, 143.6, 144.4, 148.8. IR [cm^{-1}]: 3070, 2988, 1755, 1719, 1337, 1216, 760. MS (ESI, *m/z*) 451.2 $[M + H]^+$. Anal. Calcd. for $C_{25}H_{18}N_6O_3$ (450.5): C 66.66, H 4.03, N 18.66. Found: C 66.63, H 4.11, N 18.50.

*5.1.3.2. 2-(4-Methylphenyl)-4-(1-ethoxycarbonylbenzimidazol-2-yl)-1,2-dihydro[1,2,4]triazino[4,5-a]benzimidazol-1-one (**5b**).* Yield 79.4% (method A), 72.3 (method B), beige crystals, mp 218–220 °C. ^{1}H NMR ($CDCl_3$): δ 1.25 (t, 3H, CH_3), 2.44 (s, 3H, CH_3), 4.39 (q, 2H, CH_2), 7.34 (d, 2H, arom, $J = 8.7$ Hz), 7.44–7.55 (m, 2H, arom), 7.60 (m, 4H, arom), 7.91–7.99 (m, 2H, arom), 8.07 (d, 1H, arom, $J = 7.5$ Hz), 7.55 (t, 1H, arom, $J = 4.7$ Hz). IR [cm^{-1}]: 3093, 2982, 1762, 1722, 1512, 1333, 1212, 767. MS (ESI, *m/z*) 465.2 $[M + H]^+$. Anal. Calcd. for $C_{26}H_{20}N_6O_3$ (464.5): C 67.23, H 4.34, N 18.09. Found: C 67.22, H 4.24, N 18.15.

*5.1.3.3. 2-(4-Methoxyphenyl)-4-(1-ethoxycarbonylbenzimidazol-2-yl)-1,2-dihydro[1,2,4]triazino[4,5-a]benzimidazol-1-one (**5c**).* Yield 81.3% (method A), 70.8 (method B), beige crystals, mp 214–216 °C. ^{1}H NMR ($CDCl_3$): δ 1.26 (t, 3H, CH_3), 3.88 (s, 3H, OCH_3), 4.39 (q, 2H, CH_2), 7.05 (d, 2H, arom, $J = 9.3$ Hz), 7.34–7.54 (m, 2H, arom), 7.60–7.66 (m, 4H, arom), 7.90–7.98 (m, 2H, arom), 8.07 (d, 1H, arom, $J = 7.5$ Hz), 8.50 (t, 1H, arom, $J = 5.0$ Hz). IR [cm^{-1}]: 3060, 2980, 1751, 1719, 1510, 1244, 1212, 762. MS (ESI, *m/z*) 481.1 $[M + H]^+$. Anal. Calcd. for $C_{26}H_{20}N_6O_4$ (480.5): C 64.99, H 4.20, N 17.49. Found: C 65.07, H 4.31, N 17.33.

*5.1.3.4. 2-(4-Chlorophenyl)-4-(1-ethoxycarbonylbenzimidazol-2-yl)-1,2-dihydro[1,2,4]triazino[4,5-a]benzimidazol-1-one (**5d**).* Yield 75.4% (method A), 68.1 (method B), beige crystals, mp 220–222 °C. ^{1}H NMR ($CDCl_3$): δ 1.29 (t, 3H, CH_3), 4.41 (q, 2H, CH_2), 7.48–7.55 (m, 4H, arom), 7.63–7.65 (m, 2H, arom), 7.74 (d, 2H, arom, $J = 8.7$ Hz), 7.92–7.99 (m, 2H, arom), 8.08 (d, 1H, arom, $J = 7.8$ Hz), 8.54 (t, 1H, arom, $J = 4.8$ Hz). IR [cm^{-1}]: 3088, 2980, 1759, 1725, 1335, 1207, 768. MS (ESI, *m/z*) 485.1 $[M + H]^+$. Anal. Calcd. for $C_{25}H_{17}ClN_6O_3$ (484.9): C 61.93, H 3.53, N 17.33. Found: C 61.76, H 3.76, N 17.20.

*5.1.3.5. 2-(4-Nitrophenyl)-4-(1-ethoxycarbonylbenzimidazol-2-yl)-1,2-dihydro[1,2,4]triazino[4,5-a]benzimidazol-1-one (**5e**).* Yield 72.4% (method A), 65.7 (method B), beige crystals, mp 138–142 °C dec. ^{1}H NMR ($CDCl_3$): δ 1.33 (t, 3H, CH_3), 4.43 (q, 2H, CH_2), 7.46–7.59 (m, 2H, arom), 7.64–7.67 (m, 2H, arom), 7.94–7.98 (m, 2H, arom), 8.06–8.10 (m, 3H, arom), 8.41 (d, 2H, arom, $J = 8.4$ Hz), 8.54 (t, 1H, arom, $J = 4.5$ Hz). IR [cm^{-1}]: 3094, 2984, 1755, 1724, 1523, 1344, 1213, 754. MS (ESI, *m/z*) 496.2 $[M + H]^+$. Anal. Calcd. for $C_{25}H_{17}N_7O_5$ (495.5): C 60.61, H 3.46, N 19.79. Found: C 60.38, H 3.70, N 19.88.

*5.1.4. 2-Aryl-4-(benzimidazol-2-yl)-1,2-dihydro[1,2,4]-triazino[4,5-a]benzimidazol-1-ones (**6a–6e**): general procedure*

A solution of compound **5** (0.2 mmol) in ethanol (3 ml) and hydrochloric acid (35%; 3 ml) was refluxed for 30 min, with precipitation from the solution. The mixture was diluted with water (30 ml) then precipitated solid was filtered off and washed with water. The sample for analysis was obtained by crystallization from ethanol (about 1 mg per 3 ml ethanol). The purity of these compounds was checked by TLC on silica gel plate using a chloroform–methanol mixture (80:2 v/v) as the mobile phase.

*5.1.4.1. 2-Phenyl-4-(benzimidazol-2-yl)-1,2-dihydro[1,2,4]-triazino[4,5-a]benzimidazol-1-one (**6a**).* Yield 93.8%, pale yellow needle, mp 331–333 °C. ^{1}H NMR (DMSO-d_6): δ 7.26–7.36 (m, 2H, arom), 7.51–7.75 (m, 6H, arom), 7.80–7.83 (m, 3H, arom), 8.17 (d, 1H, arom, $J = 7.2$ Hz), 8.45 (d, 1H, arom, $J = 7.2$ Hz), 13.04 (bs, 1H, NH); ^{13}C NMR (DMSO-d_6): δ 112.5, 115.1, 119.6, 120.7, 122.2, 123.7, 125.7, 126.4, 126.6, 128.4, 128.7, 129.5, 131.0, 134.4, 140.2, 140.7, 143.4, 143.5, 144.1, 144.6. IR [cm^{-1}]: 3309, 1713, 1366, 1220, 755. MS (ESI, *m/z*) 379.1 $[M + H]^+$. Anal. Calcd. for $C_{22}H_{14}N_6O$ (378.4): C 69.83, H 3.73, N 22.21. Found: C 69.78, H 3.66, N 22.16.

*5.1.4.2. 2-(4-Methylphenyl)-4-(benzimidazol-2-yl)-1,2-dihydro-[1,2,4]triazino[4,5-a]benzimidazol-1-one (**6b**).* Yield 92.4%, pale yellow needle, mp 340–342 °C. ^{1}H NMR (DMSO-d_6): δ 2.44 (s, 3H, CH_3), 7.25–7.36 (m, 2H, arom), 7.44 (d, 2H, arom, $J = 8.1$ Hz), 7.67–7.75 (m, 5H, arom), 7.81 (d, 1H, arom, $J = 8.1$ Hz), 8.16 (d, 1H, arom, $J = 7.0$ Hz), 8.47 (d, 1H, arom, $J = 7.0$ Hz), 13.03 (bs, 1H, NH). IR [cm^{-1}]: 3282, 1713, 1511, 1368, 1221, 743. MS (ESI, *m/z*) 393.1 $[M + H]^+$. Anal. Calcd. for $C_{23}H_{16}N_6O$ (392.4): C 70.40, H 4.11, N 21.42. Found: C 70.34, H 4.19, N 21.36.

*5.1.4.3. 2-(4-Methoxyphenyl)-4-(benzimidazol-2-yl)-1,2-dihydro-[1,2,4]triazino[4,5-a]benzimidazol-1-one (**6c**).* Yield 91.2%, pale yellow needle, mp 324–326 °C. ^{1}H NMR (DMSO-d_6): δ 3.87 (s, 3H, OCH_3), 7.16 (d, 2H, arom, $J = 8.7$ Hz), 7.29–7.20 (m, 2H, arom), 7.65–7.78 (m, 6H, arom), 8.15 (d, 1H, arom, $J = 7.2$ Hz), 8.44 (d, 1H, arom, $J = 7.2$ Hz), 13.03 (bs, 1H, NH). IR [cm^{-1}]: 3324, 1714, 1510, 1219, 757. MS (ESI, *m/z*) 409.1 $[M + H]^+$. Anal. Calcd. for $C_{23}H_{16}N_6O_2$

(408.4): C 67.64, H 3.95, N 20.58. Found: C 67.55, H 4.06, N 20.73.

*5.1.4.4. 2-(4-Chlorophenyl)-4-(benzimidazol-2-yl)-1,2-dihydro-[1,2,4]triazino[4,5-a]benzimidazol-1-one (**6d**).* Yield 96.0%, pale yellow needle, mp 345–347 °C. ^{1}H NMR (DMSO-d_6): δ 7.33–7.36 (m, 2H, arom), 7.68–7.73 (m, 5H, arom), 7.78–7.81 (m, 2H, arom), 7.87 (d, 1H, arom, $J = 9.0$ Hz), 8.17 (d, 1H, arom, $J = 7.0$ Hz), 8.44 (d, 1H, arom, $J = 7.0$ Hz), 13.04 (bs, 1H, NH). IR [cm^{-1}]: 3337, 1719, 1489, 1219, 739. MS (ESI, *m/z*) 413.1 $[M + H]^+$. Anal. Calcd. for $C_{22}H_{13}ClN_6O$ (412.8): C 64.01, H 3.17, N 20.36. Found: C 64.13, H 3.11, N 20.22.

*5.1.4.5. 2-(4-Nitrophenyl)-4-(benzimidazol-2-yl)-1,2-dihydro-[1,2,4]triazino[4,5-a]benzimidazol-1-one (**6e**).* Yield 86.6%, lemon yellow needle, mp over 360 °C. ^{1}H NMR (DMSO-d_6): δ 7.32–7.36 (m, 2H, arom), 7.0–7.73 (m, 2H, arom), 7.79–7.82 (m, 4H, arom), 8.17 (d, 2H, arom, $J = 9.0$ Hz), 8.49 (d, 2H, arom, $J = 9.0$ Hz), 13.08 (bs, 1H, NH). IR [cm^{-1}]: 3314, 1715, 1524, 1353, 1219, 738. MS (ESI, *m/z*) 424.1 $[M + H]^+$. Anal. Calcd. for $C_{22}H_{13}N_7O_3$ (423.4): C 62.41, H 3.09, N 23.16. Found: C 62.39, H 2.96, N 23.10.

*5.1.5. Ethyl 13-oxo-5H,13H-pyrimido[1,6-a:3,4-a′]bis-(benzimidazol)-5-carboxylate (**7**)*

A solution of compound **2** (432 mg; 1.1 mmol) in pyridine (3 ml) and water (1.5 ml) was refluxed for 60 min. The mixture was diluted with water (15 ml) and the precipitated solid was filtered off, washed with water and dried. The crude product (330 mg) was dissolved in chloroform (10 ml) and filtered through Celite. The filtered solution was evaporated under vacuum and the residue was crystallized from ethanol. Yield 250 mg (65.6%, colorless crystals), mp 175–178 °C. ^{1}H NMR ($CDCl_3$): δ 1.57 (t, 3H, CH_3), 4.60 (q, 2H, CH_2), 7.21 (s, 1H, –CH═), 7.29–7.37 (m, 3H, arom), 7.44 (t, 1H, arom $J = 7.5$ Hz), 7.72 (d, 1H, arom, $J = 8.1$ Hz), 7.96 (t, 1H, arom, $J = 5.2$ Hz), 8.39 (d, 1H, arom, $J = 8.1$ Hz), 8.45 (t, 1H, arom, $J = 6.3$ Hz); ^{13}C NMR ($CDCl_3$): δ 14.9, 65.5, 81.3, 115.5, 115.7, 115.9, 119.1, 123.3, 125.9, 126.5, 126.8, 128.4, 130.1, 130.4, 141.3, 143.6, 145.6, 149.7, 150.5. IR [cm^{-1}]: 3124, 2968, 1740, 1645, 1550, 1417, 1285, 748. MS (ESI, *m/z*) 347.1 $[M + H]^+$. Anal. Calcd. for $C_{19}H_{14}N_4O_3$ (346.3): C 65.89, H 4.07, N 16.18. Found: C 65.54, H 4.18, N 16.01.

*5.1.6. 5H,13H-Pyrimido[1,6-a:3,4-a′]bis(benzimidazol)-13-one (**8**)*

A solution of compound **7** (25.6 mg, 0.074 mmol) in ethanol (2 ml) and HCl (35%, 2 ml) was refluxed for 1 h. The pH of the cooled solution was raised to 10–11 by adding a solution of NaOH. The precipitated solid was filtered off and washed with water. Yield 15.8 mg (78.3%, colorless solid), mp over 360 °C. ^{1}H NMR (DMSO-d_6): δ 6.16 (s, 1H, –CH═), 7.28 (t, 2H, arom $J = 7.5$ Hz), 7.41 (t, 2H, arom $J = 7.8$ Hz), 7.47 (d, 2H, arom, $J = 7.5$ Hz), 8.34 (d, 2H, arom, $J = 8.1$ Hz), N–H proton is missing in the spectrum. IR [cm^{-1}]: 3108, 1714, 1660, 1564, 1251, 779. MS (ESI, *m/z*) 275.2 $[M + H]^+$. Anal. Calcd. for $C_{16}H_{10}N_4O$ (274.3): C 70.07, H 3.67, N 20.43. Found: C 69.87, H 4.01, N 20.20.

5.2. Biological activity

5.2.1. Cell lines

All cells were purchased from the American Tissue Culture Collection (ATCC), unless otherwise indicated. The daunorubicin-resistant subline of CEM cells (CEM-DNR-bulk) and paclitaxel-resistant subline K562-Tax were selected in our laboratory by cultivating the original cell lines in increasing concentrations of daunorubicin or paclitaxel, respectively [28]. The cells were maintained in Nunc/Corning 80 cm^2 plastic tissue culture flasks and cultured in DMEM/RPMI 1640 cell culture medium, with 5 g/L glucose, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 10% fetal calf serum, and $NaHCO_3$.

5.2.2. Cytotoxic MTT assay [29]

Cell suspensions were prepared and diluted according to the particular cell type and the expected target cell density (2500–30 000 cells/well based on cell growth characteristics). The cells were pipetted (80 μL) into 96-well microtiter plates, and cultured in the wells for 24 h at 37 °C in a 5% CO_2 atmosphere for stabilization. Test compounds were then added to the wells, in 20 μL portions, to the intended concentrations and the incubations continued for further 72 h at 37 °C, in a 5% CO_2 atmosphere at 100% humidity. The time they were added was defined as time zero, and each concentration was tested in duplicate. At the end of the incubation period, the numbers of viable cells were assayed using MTT. Aliquots (10 μL) of the MTT stock solution were pipetted into each well and the cells were incubated for further 1–4 h. After this incubation period the formazan produced was dissolved by adding 100 μL of 10% aq. SDS (pH = 5.5) to each well, followed by a further incubation at 37 °C overnight. The optical density (OD) of the mixture in each well was then measured at 540 nm with a Labsystem iEMS Reader MF. The tumor cell inhibitory concentration (IC) of each test compound was calculated using the following equation: $IC = (OD_{\text{drug-exposed well}}/\text{mean } OD_{\text{control wells}}) \times 100\%$. The IC_{50} value, the drug concentration lethal to 50% of the tumor cells, was calculated from appropriate dose–response curves.

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