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ORIGINAL PAPER

# Synthesis and anticancer activity of some 1,5-diaryl-3-(3,4,5-trihydroxyphenyl)-1*H*-pyrazolo[4,3-*e*][1,2,4]triazines

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**Abstract** The deregulation of cell cycle components in cancer cells has provided a rationale for the development of small molecule inhibitors of cyclin-dependent kinases as novel anticancer drugs. A series of 1,5-diaryl-3-(3,4,5-trihydroxyphenyl)-1*H*-pyrazolo[4,3-*e*][1,2,4]triazines was synthesized and their kinase inhibitory activity and cytotoxicity against several cancer cell lines has been evaluated. Some of the compounds of the series exhibited induction of caspase-dependent cell death and inhibition of cyclin-dependent kinase 2 (CDK2).

**Keywords** Pyrazolo[4,3-*e*][1,2,4]triazine · Anticancer · Cyclin-dependent kinase · Inhibitor · Caspase · Apoptosis

## Introduction

The biological activity of 1,2,4-triazine heterocyclic systems condensed with other five-membered heterocycles has been carefully studied because these moieties are bioisosteric with purine, a molecule with a wide range of biological activities [1, 2]. For example, purine-related pyrrolo[2,1-*f*][1,2,4]triazines (**1**) have been identified as tyrosine kinase inhibitors, a well-established platform for modern anticancer chemotherapy [3]. Some imidazo[2,1-*c*][1,2,4]triazine derivatives (**2**) have shown several times higher cytotoxicity against transformed cells compared with normal cells [4], and similarly interest in [1,2,4]triazino[4,5-*a*]benzimidazole derivatives (**3**) is continuing because of their remarkably selective anticancer activity [5] (Fig. 1).

The pyrazolo[4,3-*e*][1,2,4]triazine system has been less studied in comparison with other condensed heterocycles which bear a 1,2,4-triazine ring. A group of naturally occurring pyrazolo[4,3-*e*][1,2,4]triazine derivatives is produced by the microorganisms of the genuses *Pseudomonas* and *Nostoc* [6, 7]. The biosynthesis, metabolism, and function of these compounds have not been studied so far although some of them showed some anticancer and antibacterial activity [8]. The moderate inhibition of purine nucleoside phosphorylase by 3-methyl-5-methylthio-1*H*-pyrazolo[4,3-*e*][1,2,4]triazine (**4**) was also described [9]. With regard to the above discussed facts, we envisage high potential of pyrazolo[4,3-*e*][1,2,4]triazine system for further study of synthesis, reactivity, and biological activity (Fig. 2).

Our previous work [10] focused on the structure–cytotoxic activity relationship of a newly synthesized series of 1,5-diaryl-3-(3,4,5-trimethoxyphenyl)-1*H*-pyrazolo[4,3-*e*][1,2,4]triazines (**5**). Some of them exhibited relatively selective cytotoxic activity in the A549 lung carcinoma cell line at submicromolar range, while they were generally less active against other cancer cell lines, including the otherwise highly chemosensitive T cell leukemic line CEM.

In this work we describe protein kinase inhibitory and cytotoxic activities of analogous 1,5-diaryl-3-(3,4,5-trihydroxyphenyl)-1*H*-pyrazolo[4,3-*e*][1,2,4]triazines (**6**), which were prepared by the demethylation of corresponding 1,5-diaryl-3-(3,4,5-trimethoxyphenyl)-1*H*-pyrazolo[4,3-*e*]-[1,2,4]triazines (**5**) (Fig. 3). We have also analyzed cellular

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**Fig. 1** 1,2,4-Triazine scaffolds with potential as as novel anticancer drugs

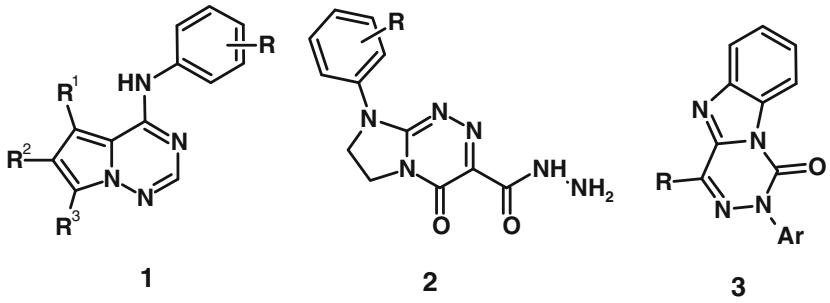
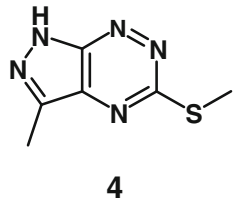



**Fig. 2** 3-Methyl-5-methylthio-1*H*-pyrazolo[4,3-*e*]-[1,2,4]triazine (**4**) is an inhibitior of purine nucleoside phosphorylase



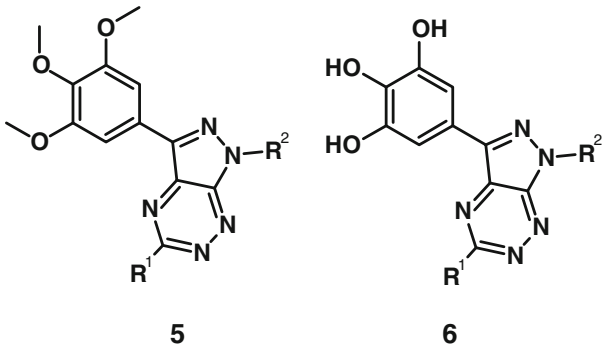




**Fig. 3** Demethylation of 1,5-diaryl-3-(3,4,5-trimethoxyphenyl)-1*H*-pyrazolo[4,3-*e*][1,2,4]triazines (**5**) provides the corresponding 3,4,5-trihydroxyphenyl variants (**6**)

and biochemical effects of selected substituted pyrazolo[4,3-*e*][1,2,4]triazines in order to better understand their molecular mechanism of action.

## Results and discussion

### *Chemistry*

The target 1,5-diaryl-3-(3,4,5-trihydroxyphenyl)-1*H*-pyrazolo[4,3-*e*][1,2,4]triazines (**6**) were prepared from analogous 1,5-diaryl-3-(3,4,5-trimethoxyphenyl)-1*H*-pyrazolo-[4,3-*e*][1,2,4]triazines (**5**) [10] by the action of boron tribromide under mild conditions in nearly quantitative yield. The newly synthesized compounds were characterized by using $^1$H NMR spectrometry, ESI–mass spectrometry, and elemental analysis. Table 1 summarizes the synthesized 1,5-diaryl-3-(3,4,5-trihydroxyphenyl)-1*H*-pyrazolo-[4,3-*e*][1,2,4]triazines (**6**).

**Table 1** Synthesized 1,3,5-trisubstituted pyrazolo[4,3-*e*][1,2,4]triazines

| Compound | $R^1$ | $R^2$ |
|---|---|---|
| **6a** | Phenyl | 2-Hydroxyphenyl |
| **6b** | Phenyl | 3-Hydroxyphenyl |
| **6c** | Phenyl | 4-Hydroxyphenyl |
| **6d** | 4-Chlorophenyl | 2-Hydroxyphenyl |
| **6e** | 4-Chlorophenyl | 3-Hydroxyphenyl |
| **6f** | 4-Chlorophenyl | 4-Hydroxyphenyl |
| **6g** | 4-Chlorophenyl | 4-Chlorophenyl |
| **6h** | 4-Chlorophenyl | 3,4,5-Trihydroxyphenyl |
| **6i** | 3,4,5-Trihydroxyphenyl | 3,4,5-Trihydroxyphenyl |

### *Biochemistry*

Some condensed 1,2,4-triazines have been previously identified as tyrosine kinase inhibitors [3]. Due to our long-term interest in the development of cyclin-dependent kinase (CDK) inhibitors, physicochemical properties of 1,5-diaryl-3-(3,4,5-trihydroxyphenyl)-1*H*-pyrazolo[4,3-*e*]-[1,2,4]triazines and their structural similarities to some known CDK inhibitors, we indeed tested all prepared compounds in our standardized CDK2 inhibition assay. CDKs belong to a family of Ser/Thr kinases which, in association with particular cyclins, play an irreplaceable role as regulators of the different phases of the cell division cycle. These enzymes and/or their direct regulators are frequently mutated, amplified, or deleted in malignant cells, and this observation has progressively given rise to the concept of pharmacological CDK inhibition as a potentially effective strategy to treat cancer [1, 11].

Some compounds of the series were able to inhibit activity of CDK2 in a biochemical assay with $IC_{50}$ values comparable to olomoucine [12]. The most active compounds bear hydroxylated phenyls at position 1, such as 3-hydroxyphenyl derivative **6b** or the 3,4,5-trihydroxyphenyl derivative **6i**. In addition we have observed that a 4-chlorophenyl side chain at position 5 significantly decreases CDK2 inhibitory activity (cf. pairs **6e** and **6b**, **6i** and **6h**).

The data on CDK2 inhibition of 1,5-diaryl-3-(3,4,5-trihydroxyphenyl)-1*H*-pyrazolo[4,3-*e*][1,2,4]triazines (**6**) are summarized in (Table 2).

*Cytotoxic activity*

The cytotoxic activities were examined under in vitro conditions using MTT assays towards five cancer cell lines: CEM (T lymphoblastic leukemia), CEM DNR bulk (T lymphoblastic leukemia, daunorubicin-resistant clone), K562 (myeloid leukemia), K562-tax (myeloid leukemia, paclitaxel-resistant clone), and A549 (lung adenocarcinoma) [10]. The cytotoxicities of compounds **6a–6i** are summarized in Table 3. Interestingly, we did not observe selectivity towards the A549 cell line like in the case of analogous 1,5-diaryl-3-(3,4,5-trimethoxyphenyl)-1*H*-pyrazolo[4,3-*e*][1,2,4]triazines (**5**) that we described recently [13] and the tested compounds (**6**) induced cytotoxicity equally in all drug-sensitive cell lines.

**Table 2** Results of CDK2 inhibition tests ($IC_{50}$ in μmol/dm$^3$)

| Compound | $IC_{50}$ CDK2 ± SD (μM) |
|---|---|
| **6a** | 15.1 ± 5.3 |
| **6b** | 5.2 ± 1.73 |
| **6c** | 14.1 ± 1.9 |
| **6d** | >20 |
| **6e** | 17.3 ± 3.5 |
| **6f** | >20 |
| **6g** | >20 |
| **6h** | 11.0 ± 4.5 |
| **6i** | 4.5 ± 2.8 |
| Olomoucine[a] | 5.0 ± 1.0 |

The presented data represent mean values from three independent experiments plus the standard deviation (SD)

[a] Olomoucine value is included as a control [12]

**Table 3** Results of MTT cytotoxic activity tests ($IC_{50}$ in μmol/dm$^3$)

| Compound | $IC_{50}$ (μM) | | | | | |
|---|---|---|---|---|---|---|
| | CEM | CEM DNR bulk | K562 | K562-tax | A549 | MCF |
| **6a** | 34 | >100 | 46 | >100 | >100 | >100 |
| **6b** | 39 | >100 | 13 | 54 | 78 | NT |
| **6c** | 12 | >100 | 23 | >100 | >100 | NT |
| **6d** | 10.1 | >100 | 40 | 32 | 12.0 | NT |
| **6e** | 1.9 | 62 | 2.4 | 56 | 43 | 5.5 |
| **6f** | 8.4 | 46 | 8.5 | 32 | 31 | 34 |
| **6g** | >100 | >100 | >100 | >100 | >100 | >100 |
| **6h** | 0.77 | 41 | 1.4 | 55 | 2.5 | 2.3 |
| **6i** | >100 | >100 | >100 | >100 | >100 | >100 |

The presented data represent mean values from three independent experiments; the standard deviation did not exceed 15% of the mean

*NT* not tested

Compounds with the highest cytotoxicity on our panel of cell lines, i.e. **6e** and **6h**, bear a 4-chlorophenyl moiety at the position 5 of the pyrazolo[4,3-*e*][1,2,4]triazine skeleton, which is in contrast to CDK2 activity, where this hydrophobic substitution decreased the ability to inhibit the kinase. In summary, the tested compounds influenced all cell lines approximately equipotently, with exception of the drug-resistant subclones of CEM and K562 cell lines. The decrease of their sensitivity might be due to increased expression of multidrug-resistance (MDR) responsible genes, such as multidrug-resistance protein 1 (MRP-1) in CEM DNR bulk or P-glycoprotein (Pgp) in K562-tax [10].

Most CDK inhibitors exert strong antiproliferative and pro-apoptotic effects [14–18]. We have therefore studied cellular and biochemical effects of compound **6h** to elucidate the molecular mechanism of action. Flow cytometric measurements of cell cycle in CEM cells treated with **6h** revealed only minor changes in cell cycle distribution, with moderate accumulation of cells in S phase and reduction of G2/M population to half its initial value (Fig. 4a).

The observed cell cycle changes could, however, result from accumulation of sub-G1 population that is generally considered as apoptotic. Dose-dependent increase of apoptotic (sub-G1) cells was further verified via induction of programmed cell death in a biochemical assay of caspase-3/7 activity. As shown in Fig. 4b, 2 μM **6h** induced strong activation of caspases-3/7 in treated cells.

Overall results suggest that the target for this class of compounds is related to proliferation and viability. One of the possible underlying mechanisms can be directly caused by inhibition of CDKs, as these enzymes regulate not only the cell cycle, but also transcription and hence viability of cells. During the last 20 years, a number of CDK inhibitors have been developed and some of them have already entered clinical trials as antitumor agents [2]. The prepared pyrazolo[4,3-*e*][1,2,4]triazines can therefore be considered as a novel scaffold for development of antiproliferative agents with possible pharmacological applications in oncology.

## Experimental

Melting points were determined on a Boetius stage and are corrected. $^1$H NMR spectra were measured in DMSO-$d_6$ at 300 K on a Bruker Avance 300 spectrometer (300 MHz) with TMS as an internal standard; chemical shifts are reported in ppm, and coupling constants in Hz. Mass spectra were recorded by using an LCQ ion trap mass spectrometer (Finnigan MAT, San Jose, CA, USA). Elemental analyses were performed by using an EA 1108

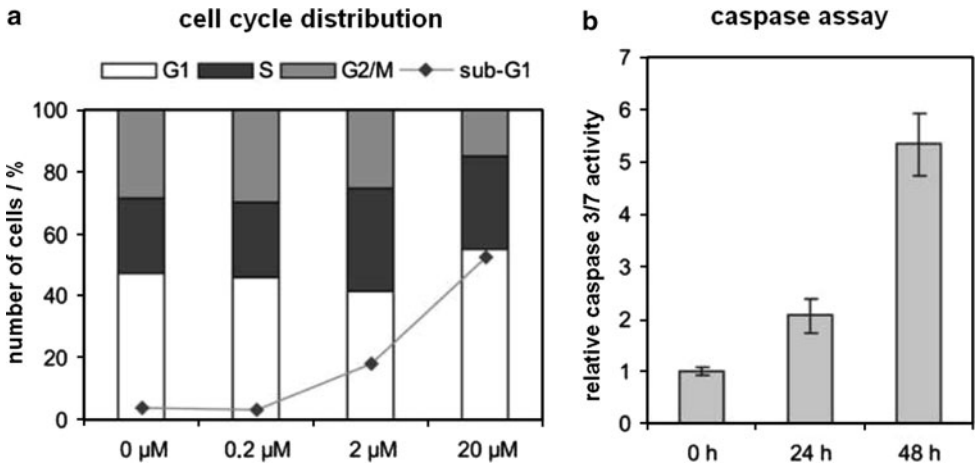



**Fig. 4** Effect of compound **6h** on the cell cycle profile within 24 h (**a**) and activation of an apoptotic marker caspase-3/7 within 24 and 48 h (**b**) in cell line CEM

Elemental Analyzer (Fison Instruments); their values (C, H, N) agreed with the calculated ones within acceptable limits.

*General procedure for synthesis of 1,5-diaryl-3-(3,4,5-trihydroxyphenyl)-1H-pyrazolo[4,3-e][1,2,4]triazines*

To a stirred solution of 1,5-diaryl-3-(3,4,5-trimethoxyphenyl)-1*H*-pyrazolo[4,3-*e*][1,2,4]triazine (1.00 mmol) in 25 $cm^3$ dry dichloromethane was added a solution of boron tribromide (1.05 mmol for each methoxy group) in 25 $cm^3$ dry dichloromethane during 5 min at room temperature under argon atmosphere. The reaction mixture was stirred under an argon atmosphere at room temperature for a further 24 h. The reaction mixture was then filtered through Celite and 10 $cm^3$ methanol was added to the filtrate. The resulting solution was evaporated under reduced pressure and the resulting solid was suspended in 10 $cm^3$ water, filtered off, and washed three times with 5 $cm^3$ water. The crude product was dried in a vacuum desiccator and purified by flash chromatography using chloroform–methanol (4:1, v/v) as mobile phase.

*1-(2-Hydroxyphenyl)-5-phenyl-3-(3,4,5-trihydroxyphenyl)-1H-pyrazolo[4,3-e][1,2,4]triazine* (**6a**, $C_{22}H_{15}N_5O_4$)
Yield: 88%; m.p.: 275–277 °C; $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta = 7.07$ (1H, t, $J = 7.44$ Hz), 7.19 (1H, d, $J = 7.44$ Hz), 7.47 (1H, t, $J = 7.44$ Hz), 7.59–7.68 (6H, m), 8.66 (2H, d, $J = 6.78$ Hz) ppm; $^{13}$C NMR (75 MHz, DMSO-$d_6$): $\delta = 110.3$, 111.5, 118.5, 118.9, 119.6, 124.8, 127.5, 128.9, 144.5, 149.7, 155.6, 155.8, 159.4, 160.2, 161.4, 163.6, 165.7, 166.8 ppm; ESI–MS: *m/z* (%) = 414 ($M^+$, 98).

*1-(3-Hydroxyphenyl)-5-phenyl-3-(3,4,5-trihydroxyphenyl)-1H-pyrazolo[4,3-e][1,2,4]triazine* (**6b**, $C_{22}H_{15}N_5O_4$)
Yield: 92%; m.p.: >360 °C; $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta = 7.26$ (1H, t, $J = 7.8$ Hz), 7.41 (1H, d, $J = 7.8$ Hz), 7.63–7.68 (4H, m), 7.73 (1H, d, $J = 7.8$ Hz), 7.85 (2H, s), 8.58 (2H, d, $J = 7.8$ Hz) ppm; $^{13}$C NMR (75 MHz, DMSO-$d_6$): $\delta = 106.5$, 107.4, 118.5, 118.8, 120.5, 122.8, 124.6, 128.9, 142.0, 144.6, 155.6, 155.8, 160.0, 160.4, 162.4, 163.6, 164.2, 166.8 ppm; ESI–MS: *m/z* (%) = 414 ($M^+$, 99).

*1-(4-Hydroxyphenyl)-5-phenyl-3-(3,4,5-trihydroxyphenyl)-1H-pyrazolo[4,3-e][1,2,4]triazine* (**6c**, $C_{22}H_{15}N_5O_4$)
Yield: 78%; m.p.: >360 °C; $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta = 7.07$ (1H, t, $J = 7.44$ Hz), 7.19 (2H, s), 7.59–7.68 (6H, m), 8.66 (2H, d, $J = 7.86$ Hz) ppm; $^{13}$C NMR (75 MHz, DMSO-$d_6$): $\delta = 112.4$, 114.5, 118.9, 119.6, 122.6, 129.0, 144.5, 149.7, 152.3, 154.8, 159.4, 160.5, 161.0, 162.4, 166.0, 166.8 ppm; ESI–MS: *m/z* (%) = 414 ($M^+$, 97).

*5-(4-Chlorophenyl)-1-(2-hydroxyphenyl)-3-(3,4,5-trihydroxyphenyl)-1H-pyrazolo[4,3-e][1,2,4]triazine*
(**6d**, $C_{22}H_{14}N_5O_4Cl$)
Yield: 87%; m.p.: 279–281 °C; $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta = 6.90$ (2H, s), 7.25 (1H, t, $J = 7.90$ Hz), 7.39 (1H, t, $J = 7.90$ Hz), 7.58 (2H, d, $J = 7.65$ Hz), 7.65–7.74 (2H, m), 8.08 (2H, d, $J = 7.65$ Hz) ppm; $^{13}$C NMR (75 MHz, DMSO-$d_6$): $\delta = 111.0$, 116.5, 117.3, 123.6, 124.8, 128.9, 147.5, 152.1, 155.6, 155.8, 159.4, 161.2, 161.8, 163.0, 165.3, 169.0 ppm; ESI–MS: *m/z* (%) = 448 ($M^+$, 96).

*5-(4-Chlorophenyl)-1-(3-hydroxyphenyl)-3-(3,4,5-trihydroxyphenyl)-1H-pyrazolo[4,3-e][1,2,4]triazine*
(**6e**, $C_{22}H_{14}N_5O_4Cl$)
Yield: 67%; m.p.: 238–240 °C; $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta = 6.48$ (2H, s), 6.71 (1H, s), 7.39 (1H, t, $J = 7.90$ Hz), 7.58 (2H, d, $J = 7.65$ Hz), 7.65–7.74 (2H, m), 8.08 (2H, d, $J = 7.65$ Hz) ppm; $^{13}$C NMR (75 MHz, DMSO-$d_6$): $\delta = 108.1$, 116.0, 117.5, 119.0, 119.8, 122.0, 124.3, 133.6, 144.7, 151.9, 157.2, 158.8, 159.3, 162.2, 163.4, 166.6, 168.7, 166.8 ppm; ESI–MS: *m/z* (%) = 448 ($M^+$, 98).

*5-(4-Chlorophenyl)-1-(4-hydroxyphenyl)-3-(3,4,5-trihydroxyphenyl)-1H-pyrazolo[4,3-e][1,2,4]triazine* (**6f**, $C_{22}H_{14}N_5O_4Cl$)
Yield: 92%; m.p.: >360 °C; $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ = 7.64–7.80 (4H, m), 7.76 (2H, d, $J$ = 8.76 Hz), 8.42 (2H, d, $J$ = 8.76 Hz), 8.68 (2H, d, $J$ = 7.65 Hz) ppm; $^{13}$C NMR (75 MHz, DMSO-$d_6$): $\delta$ = 115.1, 117.6, 122.0, 123.7, 124.1, 133.0, 148.5, 150.0, 152.3, 155.0, 159.4, 160.5, 161.9, 164.4, 166.7, 169.0 ppm; ESI–MS: $m/z$ (%) = 448 ($M^+$, 100).

*1,5-Bis(4-chlorophenyl)-3-(3,4,5-trihydroxyphenyl)1-H-pyrazolo[4,3-e][1,2,4]triazine* (**6g**, $C_{22}H_{13}N_5O_3Cl_2$)
Yield: 82%; m.p.: 291–292 °C; $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ = 7.62–7.76 (4H, m), 7.76 (2H, d, $J$ = 8.76 Hz), 8.45 (2H, d, $J$ = 8.50 Hz), 8.62 (2H, d, $J$ = 8.50 Hz) ppm; $^{13}$C NMR (75 MHz, DMSO-$d_6$): $\delta$ = 113.9, 114.1, 122.0, 122.3, 126.6, 132.4, 141.6, 150.0, 154.3, 154.8, 159.4, 160.3, 161.0, 161.3, 165.8, 168.7 ppm; ESI–MS: $m/z$ (%) = 467 ($M^+$, 98).

*5-(4-Chlorophenyl)-1,3-bis(3,4,5-trihydroxyphenyl)-1H-pyrazolo[4,3-e][1,2,4]triazine* (**6h**, $C_{22}H_{14}N_5O_6Cl$)
Yield: 84%; m.p.: >360 °C; $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ = 6.44 (2H, s), 6.48 (2H, s), 7.53 (2H, d, $J$ = 8.50 Hz), 8.01 (2H, d, $J$ = 8.50 Hz) ppm; $^{13}$C NMR (75 MHz, DMSO-$d_6$): $\delta$ = 115.3, 118.9, 122.1, 122.6, 129.0, 138.7, 144.5, 149.7, 152.3, 154.8, 159.7, 160.0, 161.0, 161.9, 165.4, 169.0 ppm; ESI–MS: $m/z$ (%) = 480 ($M^+$, 99).

*1,3,5-Tris(3,4,5-trihydroxyphenyl)-1H-pyrazolo[4,3-e][1,2,4]triazine* (**6i**, $C_{22}H_{15}N_5O_9$)
Yield: 23%; m.p.: 128–130 °C; $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ = 6.42 (2H, s), 6.52 (2H, s), 7.18 (2H, s) ppm; $^{13}$C NMR (75 MHz, DMSO-$d_6$): $\delta$ = 112.4, 112.7, 113.3, 139.6, 142.7, 145.0, 147.5, 151.3, 152.0, 155.8, 156.3, 158.3, 161.6, 162.0, 166.0, 168.9 ppm; ESI–MS: $m/z$ (%) = 494 ($M^+$, 99).

### *MTT cytotoxic assay*

The assay was performed according to a published procedure [10, 13]. Briefly, cells were added into 96-well microtiter plates. Incubation of the cells with the test compounds lasted for 72 h at 37 °C in a 5% $CO_2$ atmosphere at 100% humidity. At the end of this period, MTT stock solution was pipetted into each well and incubated for 1 h. After this incubation period, formazan produced was dissolved and its absorbance was measured at 540 nm with a Labsystem iEMS Reader MF after 24 h. The $IC_{50}$ value, the drug concentration lethal to 50% of the tumor cells, was calculated from appropriate dose–response curves.

### *CDK2 inhibition assay*

CDK2-cyclin E kinase was expressed and assayed as previously described [12, 19]. Briefly, the enzyme produced in Sf9 cells was purified on an NiNTA column and assayed with histone H1 and 15 μM [$\gamma$-$^{33}$P]ATP. Kinase activity was expressed as a percentage of maximum activity. The concentration of the test compounds required to decrease the CDK activity by 50% was determined from dose–response curves and designated $IC_{50}$.

### *Cell cycle analysis*

Cell cycle analysis was performed as described previously [12]. Treated cells were collected by centrifugation, fixed with ice-cold 70% ethanol, incubated on ice for 30 min, washed with PBS, and incubated with propidium iodide (0.1 mg/cm$^3$) and RNAse A (0.5 mg/cm$^3$) for 1 h at room temperature in the dark, and finally measured on a flow cytometer equipped with a 488-nm laser (Cell Lab Quanta SC, Beckman Coulter). Analysis of cell cycle distribution was then performed by using MultiCycle AV software (Phoenix Flow Systems).

### *Caspases-3/7 assay*

Caspase assay was performed as described previously [18]. Treated cells were harvested by centrifugations and homogenized in an extraction buffer (10 mM KCl, 5 mM Hepes, 1 mM EDTA, 1 mM EGTA, 0.2% CHAPS, inhibitors of proteases, pH 7.4) on ice for 20 min. After centrifugation, lysates were incubated for 1 h with 100 μM Ac-DEVD-AMC as a caspase substrate (Sigma–Aldrich) in an assay buffer (25 mM PIPES, 2 mM EGTA, 2 mM $MgCl_2$, 5 mM DTT, pH 7.3). For negative controls, the lysates were supplemented with 100 μM Ac-DEVD-CHO as a caspase-3/7 inhibitor (Sigma–Aldrich). The fluorescence of the product was measured by using a Fluoroskan Ascent microplate reader (Labsystems) at 346 nm/442 nm (ex/em).

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