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Synthesis and hypoxic–cytotoxic activity of some 3-amino-1,2,4-benzotriazine-1,4-dioxide derivatives

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Abstract—A series of 3-amino-1,2,4-benzotriazine-1,4-dioxide derivatives **1** have been synthesized and evaluated for their cytotoxic activity in vitro against human leukemia cell lines: Molt-4, K562, HL60, human liver cancer cell Hep-G2, human prostate cancer cell PC-3 in hypoxia. Most of the compounds showed more potent activity than **TPZ**. Compounds **1i** and **1m** displayed encouraging superior activity against Molt-4 and HL-60 cell lines. Three potential derivatives received the test of the activity in hypoxia and in normoxia against Molt-4 and HL-60 cell lines and showed obvious hypoxia selectivity. Further mechanism study revealed that the cytotoxic activities of compounds **1i** and **1k** in Molt-4 cells might be mediated by modulation of p53 protein expression and mitochondrial membrane potential ($\Delta\Psi_m$).

The hypoxic cells in solid tumor lead to their resistance to radiotherapy and chemotherapy, as well as increasing the probability of tumor metastases.[1–6] However, tumor hypoxia also provides a target for the therapeutic strategies. One such strategy is to use bioreductive prodrugs that are transformed after administration under hypoxic conditions, either by metabolism or by spontaneous chemical breakdown, to form pharmacologically active species. There are four main classes of bioreductive prodrugs: nitroaromatics/heterocyclics[7], quinones, *N*-oxides,[8–11] and metal complexes.[12–15]

Among the *N*-oxides, tirapazamine (3-amine-1,2,4-benzotriazine-1,4-dioxide, SR4233, WIN 59075, **TPZ**) as one of the most promising prodrugs is in phases II and III clinical trials.[16]

Some studies indicate the diffusion ability of **TPZ** derivatives can affect the cytotoxic activity (those with low diffusion ability cannot diffuse relatively large distance from functional blood vessels to reach hypoxic tumor cells). And one of the most reliable methods for improving their diffusion ability is to increase its lipophilicity by incorporating with a properly lipophilic group.[17–20]

In addition, many previous studies reported, for the acidic atmosphere of cancer cells, the derivatives of **TPZ** with basic group in 3-position such as amino group and substituted amino group showed more activity and hypoxia selectivity.[18–20]

In order to improve the lipophilicity of TPZ and find some compounds with higher activity against tumor in hypoxia, in this article, some alkyl and aromatic groups were introduced into the amino group of 3-position in **TPZ**. We synthesized 15 known and new compounds,[21] and evaluated for their cytotoxic activity in vitro. Further study on the mechanisms of cytotoxic activity in hypoxia was also performed with two potent compounds in Molt-4 cells.

3-Amino-1,2,4-benzotriazine-1,4-dioxide derivatives **1** were prepared as shown in Scheme 1. Treatment of *o*-nitroaniline **2** with triphosgene in toluene under reflux for 3 h resulted in 2-nitrophenyl isocyanate **3**. Reacting **3** with anhydrous ammonia gas provided 2-nitrophenylurea **4**. Cyclization of **4** in 30% aq NaOH afforded the 3-hydroxy-1,2,4-benzotriazine-1-oxide **5**. Chlorination

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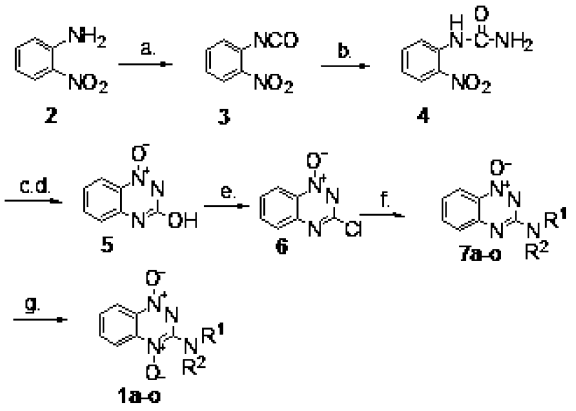


Scheme 1. The synthetic route of compounds **1a**–**o**. Reagents and conditions: (a) $(COCl_2)_3$, toluene, reflux, 3 h; (b) NH_3; (c) NaOH; (d) AcOH; (e) $POCl_3$, reflux, 3 h; (f) R^1–NH–R^2, ethanol, reflux, 12 h; (g) $AcOH/H_2O_2$, 50 °C, 24 h.

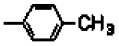
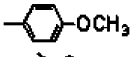
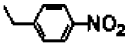
of **5** with phosphorus oxychloride produced 3-chloro-1,2,4-benzotriazine-1-oxide **6** according to the known methods with minor modifications.[22,23] Compound **6** was substituted with various primary or secondary amines to yield the 3-amino-1,2,4-benzotriazine-1-oxide **7**, which was oxidized by 30% hydrogen peroxide to afford the desired 3-amino-1,2,4-benzotriazine-1,4-dioxide **1**. The structures of compounds obtained were determined by IR, MS, NMR, and elemental analysis.

All the prepared compounds were evaluated for their cytotoxic activity in vitro against human leukemia cell lines (Molt-4, K562, and HL60), human liver cancer cell line (Hep-G2), and human prostate cancer cell line (PC-3) in hypoxia, using **TPZ** as the reference drug according to the reported methods.[24] After 48 h treatment, the cell growth rates were determined by MTT assay and the IC_{50} values were calculated with LOGIT method. The results are summarized in Table 1.

Three potential compounds **1i**, **1k**, **1m** and **TPZ** were selected to test the cytotoxicity against Molt-4 and HL-60 cell lines in normoxia or in hypoxia for 48 h. The hypoxic cytotoxicity ratio (HCR), and the IC_{50} values in normoxia and in hypoxia, was calculated. The results are presented in Table 2.

As shown in Table 1, most tested compounds exhibited higher cytotoxic activities than **TPZ** in hypoxia, in particular for leukemia cell lines. Obviously, the cytotoxic potency of **1a**–**o** against these five cancer cell lines was highly dependent on structures of the 3-alkylamino side chains. When R^1 was alkyl group or aromatic group and R^2 was hydrogen (e.g., **1a**–**1m**), they showed lower IC_{50} values than **TPZ** for most of the tested cancer cell lines. Among them, compound **1i**, when R^1 was substituted aromatic group, showed 0.6 μM and 0.9 μM IC_{50} values at the tested Molt-4 and HL-60 cell lines. Compound **1m** was the most potent cytotoxic compound against all of the tested cancer cell lines, with IC_{50} values in the range of 0.8–7.7 μM. It can be assumed that the nitro group on the benzene ring was important for the cytotoxic activities. On the other hand, compounds **1a**–**1d,** in particular **1c**, with alkylamino in 3-position were less active than

Table 1. Cytotoxicity of the target compounds against five human cancer cell lines in vitro

Compound	R^1	R^2	Cytotoxicity (IC_{50}, μM)[a]				
			Molt-4	HL-60	K562	Hep-G2	PC-3
TPZ	H	H	4.6 ± 0.3	7.0 ± 2.6	5.2 ± 0.8	19.1 ± 2.2	22.3 ± 4.7
1a	$-CH_2CH_3$	H	3.3 ± 0.8	9.0 ± 4.2	4.5 ± 2.3	13.5 ± 0.7	13.3 ± 2.6
1b	$-(CH_2)_7CH_3$–*n*	H	4.9 ± 1.5	7.2 ± 2.8	4.7 ± 0.5	17.4 ± 8.8	18.2 ± 1.7
1c	$-CH_2CH{=}CH_2$	H	3.0 ± 1.0	25.8 ± 6.0	11.2 ± 3.4	>56	>56
1d	$-CH_2CH_2OH$	H	2.6 ± 1.7	10.7 ± 1.3	3.7 ± 0.6	23.2 ± 3.1	22.4 ± 5.3
1e		H	1.9 ± 1.0	3.6 ± 1.2	6.6 ± 1.0	13.3 ± 0.7	15.9 ± 4.8
1f		H	1.3 ± 0.7	2.5 ± 1.1	2.6 ± 1.1	9.5 ± 2.1	10.5 ± 3.6
1g		H	1.4 ± 1.1	2.9 ± 0.9	2.4 ± 0.7	15.0 ± 6.4	17.0 ± 3.2
1h		H	1.1 ± 0.2	1.5 ± 0.8	2.5 ± 0.9	10.6 ± 0.5	10.3 ± 2.0
1i		H	0.6 ± 0.4	0.9 ± 0.2	2.9 ± 0.2	10.1 ± 0.5	12.0 ± 10.6
1j		H	2.3 ± 1.8	8.3 ± 2.8	10.6 ± 1.1	17.4 ± 7.4	16.1 ± 2.4
1k		H	2.2 ± 0.6	2.5 ± 0.4	1.9 ± 0.1	8.2 ± 1.3	10.7 ± 0.9
1l		H	2.3 ± 0.7	5.8 ± 1.6	2.2 ± 1.0	9.7 ± 1.5	10.6 ± 0.9
1m		H	0.8 ± 0.3	1.4 ± 0.8	3.1 ± 0.8	7.7 ± 0.8	7.4 ± 2.5
1n	$-CH_2CH_3$	$-CH_2CH_3$	>17.6	>49	>55	>107	>107
1o			>16.1	>60	>47	>95	>95

[a] Each experiment was independently performed three times and expressed as means ± SD.

Table 2. Cytotoxicity data of derivatives against Molt-4 and HL-60 cells in normoxia and in hypoxia

Compound	IC_{50}[d] (Molt-4, μM)			IC_{50}[d] (HL-60, μM)		
	Normoxia[a]	Hypoxia[b]	HCR[c]	Normoxia[a]	Hypoxia[b]	HCR[c]
TPZ	15.8 ± 3	4.6 ± 0.3	3.43	16.1 ± 3.4	7.0 ± 2.6	2.31
1i	5.2 ±1.4	0.6 ± 0.4	8.67	6.8 ± 1.3	0.9 ± 0.2	7.56
1k	8.1 ± 3.0	2.2 ± 0.6	3.68	6.3 ± 2.4	2.5 ± 0.4	2.53
1m	5.0 ± 1.7	0.8 ± 0.3	6.25	6.0 ± 2.1	1.4 ± 0.8	4.29

[a] IC_{50} values in normoxia.
[b] IC_{50} values in hypoxia (3% O_2).
[c] Hypoxic cytotoxicity ratio = IC_{50} (normoxia)/IC_{50} (hypoxia).
[d] Each experiment was independently performed three times and expressed as means ± SD.

corresponding 3-substituted phenylamino analogues **1e**–**1m**. When both of R^1 and R^2 were alkyl group, such as **1n** and **1o** it did lead to a loss of inhibitory activity. These results suggest that **1i** and **1m** might be promising cytotoxins in hypoxia and be worthy to be evaluated intensively.

Three potential compounds were more cytotoxic in hypoxia than in normoxia against Molt-4 and HL-60 cells. The HCR of these compounds against Molt-4 and HL-60 cell lines ranged from 3 to 9 (Table 2).

Further study on the mechanisms of cytotoxic activity in hypoxia was performed with two potent compounds (**1i**, **1k**) and **TPZ** as the positive drug in Molt-4 cells. Molt-4 cells were treated with tested compounds for 12 h or 24 h, respectively, then the apoptosis, mitochondrial membrane potential ($\Delta\Psi_m$), and p53 expression were determined according to the reported methods.[24] The results are shown in Figures 1–3. All experiments were repeated three times.

Molt-4 cells were cultured in complete medium with 2.0 μM **TPZ**, **1i**, and **1k** for 24 h in 3% O_2 condition. As shown in Figure 1, after 24 h, **TPZ**, **1i**, and **1k** induced apoptosis in 11%, 45% and 33%, respectively, while spontaneous apoptosis of the control (without drug) was 3%. With the same concentration, apoptotic

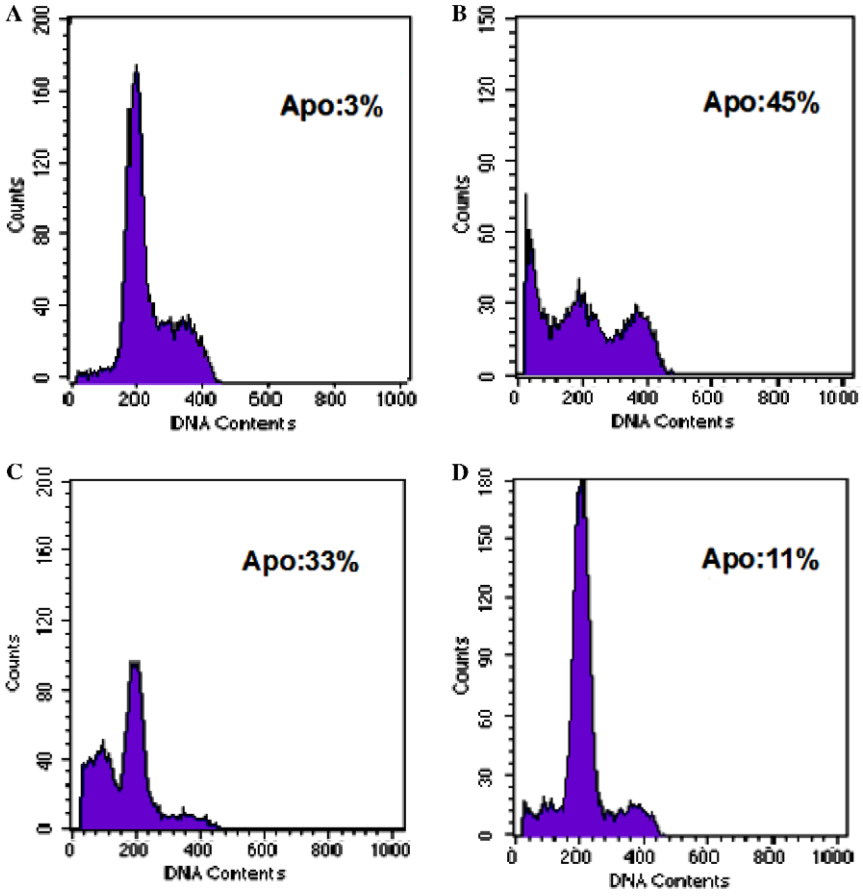


Figure 1. **TPZ**, **1k** and **1i** induced apoptosis in Molt-4 cells. Molt-4 cells were treated with 2.0 μM **TPZ**, **1k**, and **1i** in hypoxia. Without (A)/with **1i** (B), **1k** (C) or **TPZ** (D) for 24 h. Apoptosis was assessed by propidium iodide staining of lysed cell nuclei. The DNA content of 20,000 events was analyzed per test by flow cytometry. Apo, apoptosis. Two additional experiments yielded equivalent results.

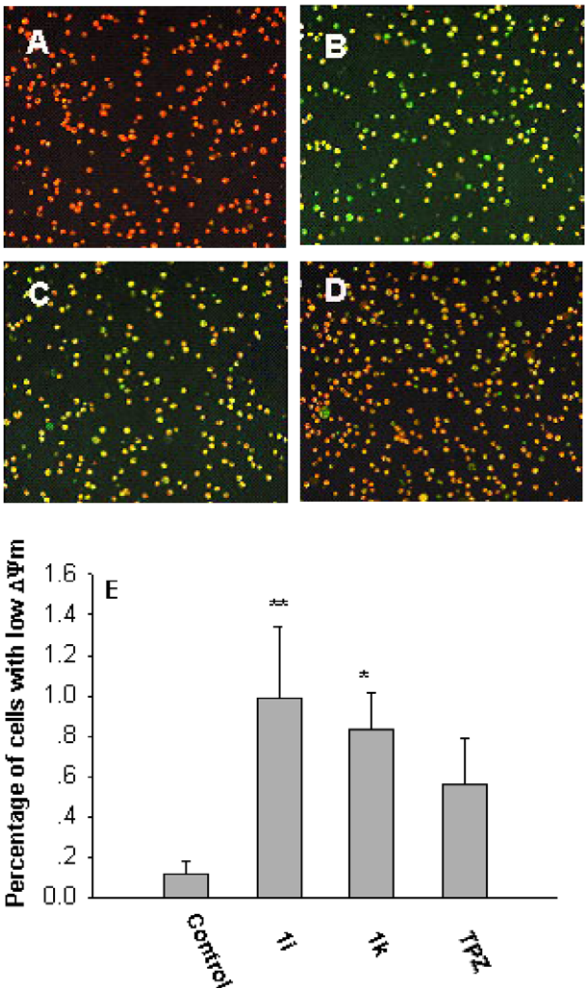


Figure 2. **TPZ**, **1i**, and **1k** induced $\Delta\Psi_m$ loss in Molt-4 cells treated with 2.0 μM **TPZ**, **1i**, and **1k** for 12 h in hypoxia. Without (A)/with **1i** (B), **1k** (C) or **TPZ** (D). $\Delta\Psi_m$ loss was assessed by JC-1 staining in which mitochondria depolarization is indicated by an increase in the green to red fluorescence intensity ratio. The quantitated data from flow cytometry are represented as a histogram (E). Values represent means ± SD for three separate experiments performed in triplicate. **, $P < 0.01$, *, $P < 0.05$ versus **TPZ** group.

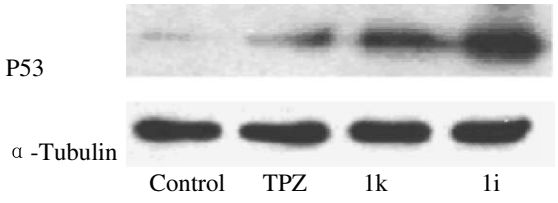


Figure 3. Induction of p53 overexpression in Molt-4 cells treated with 2.0 μM **TPZ**, **1i**, and **1k** for 24 h in hypoxia was observed. α-Tublin was shown as a loading control. Two additional experiments yielded equivalent results.

cells induced by **1i** and **1k** were significantly ($P < 0.01$) higher than that of **TPZ**, indicating the higher cytotoxic activity of **1i** and **1k** than, **TPZ**, which is consistent with the IC_{50} values.

To investigate the pathway of apoptosis induced by tested compounds, $\Delta\Psi_m$ and p53 expression in Molt-4 cells treated with 2.0 μM **TPZ**, **1i**, and **1k** for 12 h in hypoxia were determined. Loss of $\Delta\Psi_m$ could cause the release of cytochrome *C* and other factors that trigger apoptosis.[25] With JC-1 staining, mitochondria depolarization is specifically indicated by a fluorescence emission shift from red to green (and the yellow fluorescence is intermediate state). $\Delta\Psi_m$ in Molt-4 cells was reduced by **TPZ**, **1i**, and **1k** after 12 h treatment and the quantitated data from flow cytometry after JC-1 staining are shown in Figure 2, comparing with control, Molt-4 cells treated with **1i**, **1k**, and **TPZ** exhibited a mass of yellow and green fluorescence, and **1i** and **1k** showed the stronger ability in $\Delta\Psi_m$ decline than **TPZ** at the same concentration level ($P < 0.05$–0.01), suggesting that **1i** and **1k** might possess more potent cytotoxic activity through mitochondrial pathway.

P53 is involved in cell apoptosis and DNA damage. The expression of p53 in Molt-4 cells treated with **TPZ**, **li**, or **1k** was performed by Western blot analysis. As shown in Figure 3, 2.0 μM **TPZ**, **1i**, and **1k** increased p53 level in Molt-4 cells after 24 h of exposure in hypoxia. According to the data we have obtained, we confirmed that **1i** and **1k** exerted higher cytotoxicity, and the activity was related to the p53-mediated apoptosis pathway.

In summary, a series of 3-amino-1,2,4-benzotriazine-1,4-dioxide derivatives, **1a**–**o**, have been synthesized and evaluated for their cytotoxic activities in hypoxia. Most tested compounds had higher cytotoxic activities than **TPZ** in vitro, and their cytotoxic potency was highly dependent on structures of the 3-alkylamino side chains. Three tested compounds showed higher hypoxic selectivity against Molt-4 and HL-60 cell lines. Mechanism study revealed that the cytotoxic activities of these compounds might be mediated by modulation of p53 protein expression and mitochondrial membrane potential ($\Delta\Psi_m$).

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Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bmcl.2006.05.095.

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