Full Paper

# Synthesis, Structure and Hypoxic Cytotoxicity of 3-Amino-1,2,4-benzotriazine-1,4-dioxide Derivatives

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A series of novel 3-amino-1,2,4-benzotriazine-1,4-dioxide derivatives were synthesized and screened for their *in vitro* cytotoxicity against promyelocytic leukemia HL-60, androgen-independent prostate tumor PC3, hepatocellular carcinoma Bel-7402, human esophagus tumor ECA-109, and human breast tumor MCF-7 cell lines in hypoxia and in normoxia. Most compounds showed higher cytotoxic activity both in hypoxia and in normoxia. Among them, compounds **61** and **62** showed more potent cytotoxic activity and hypoxic selectivity when compared to tirapazamine.

**Keywords:** Cytotoxicity / Hypoxia / Tirapazamine

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## Introduction

Solid tumors in the hypoxic areas are more resistant to chemotherapy and radiotherapy than aerobic ones. However, hypoxia also promotes the production of a class of important and specific antitumor prodrugs, namely bioreductive agents, including quinones, nitro derivatives, and *N*-oxides [1 – 4].

Among a large number of *N*-oxides, a benzotriazine dioxide named tirapazamine, (3-amino-1,2,4-benzotriazine-1,4-dioxide, SR4233, WIN 59075, TPZ), is in phase II and III of clinical trials, alone or in combination with cisplatin-based chemotherapy and radiotherapy [5, 6]. However, some reports showed the antitumor efficacy of tirapazamine is reduced by its limited diffusion to reach all hypoxic tumor cells [7, 8]. Therefore, the discovery of new prodrugs with properly optimized biological properties is a challenge in this research area. In the previous work of our team, we found that introduction of some lipophilic groups at the C-3 amino group could improve the activity and hypoxic selectivity of *N*-oxides [9]. The present work is an extension of our ongoing efforts towards the development and identification of new molecules possessing cytotoxic activity and hypoxic selectivity. Accordingly, we have designed and synthesized a series of 3-amino-1,2,4-benzotriazine-1,4-dioxide derivatives by adding small lipophilic groups to the C-3 amino group and electron-adopting substituents or electron-donating substituents to the benzene ring of tirapazamine. The synthesized compounds were evaluated for their cytotoxicity and hypoxia selectivity *in vitro*.

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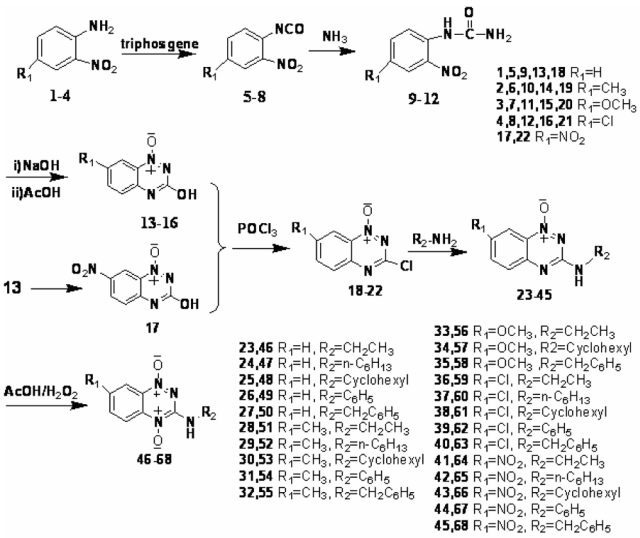
## Results and discussion

3-Amino-7-substituted-1,2,4-benzotriazine-1,4-dioxide derivatives **46 – 68** were prepared according to the known methods with minor modification as shown in Scheme 1 [9 – 11]. Treatment of *o*-nitroaniline **1 – 4** with triphosgene in toluene under reflux for 3 h resulted in 2-nitrophenyl isocyanates **5 – 8**. Reacting **5 – 8** with anhydrous ammonia gas provided 2-nitrophenylureas **9 – 12**, followed by cyclization of **9 – 12** in 30% aq-NaOH afforded the 3-hydroxy-1,2,4- benzotriazine-1-oxides **13 – 16**. 3-Hydroxy-1,2,4-benzotriazine-1-oxide **13** was nitrified by a mixture of nitric acid and vitriol to yield 3-hydroxy-7-nitro-1,2,4-benzotriazine-1-oxide **17**.




**Scheme 1**. Design and synthesis of the TPZ derivatives.

Chlorination of **13 – 17** with phosphorus oxychloride produced 3-chloro-1,2,4-benzotriazine-1-oxides **18 – 22**. Compounds **18 – 22** were substituted with various primary amines to yield the 3-amino-1,2,4-benzotriazine-1-oxides **23 – 45**, which were oxidized with 30% hydrogen peroxide to afford the desired 3-amino-1,2,4-benzotriazine-1,4-dioxides **46 – 68**. The structures of the synthesized compounds were determined by IR, MS, and NMR. A preliminary evaluation of the cytotoxic activity of all the target compounds was performed by MTT assays *in vitro*. Five cancer cell lines (HL-60: promyelocytic leukemia, BEL-7402: hepatocellular carcinoma, ECA-109: human esophagus tumor, MCF-7: human breast cancer, PC-3: androgen-independent prostate tumor) were employed in the present study. Cancer cells were seeded in 96-well microtiter plates (4000 cells/well) and maintained in hypoxia and in normoxia, respectively. TPZ was considered as the reference drug according to the literature method [12]. The $IC_{50}$ values and the hypoxic cytotoxicity ratio (HCR, the ratio of $IC_{50}$ values in normoxia / in hypoxia) are shown in Table 1 and Table 2, respectively.

As shown in Table 1, most tested compounds exhibited higher cytotoxic activities than TPZ in hypoxia toward some tested cancer cell lines. The substituents on the ben-

**Table 1**. Cytotoxicity of TPZ derivatives (**46 – 68**) against five human cancer cell lines in hypoxia and in normoxia.

| Comp. | Cytotoxicity ($IC_{50}$, μM)[a] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | HL-60 | | BEL-7402 | | ECA-109 | | MCF-7 | | PC-3 | |
| | H[b] | N[c] | H | N | H | N | H | N | H | N |
| **TPZ** | 1.6 ± 0.8 | 1.8 ± 0.6 | 1.7 ± 0.3 | 3.0 ± 0.8 | 0.7 ± 0.1 | 4.7 ± 0.8 | 2.6 ± 0.6 | 4.5 ± 0.4 | 1.2 ± 0.3 | 4.5 ± 0.1 |
| **46** | 1.8 ± 0.6 | 2.9 ± 0.9 | 1.9 ± 0.6 | 4.1 ± 0.3 | 0.8 ± 0.2 | 4.5 ± 0.4 | 2.7 ± 0.5 | 5.7 ± 0.5 | 2.0 ± 0.4 | 8.6 ± 0.4 |
| **47** | 0.6 ± 0.2 | 1.8 ± 0.6 | 3.2 ± 0.7 | 2.6 ± 0.2 | 1.7 ± 0.5 | 2.9 ± 0.9 | 2.4 ± 0.5 | 3.0 ± 0.7 | 2.7 ± 0.6 | 5.0 ± 0.9 |
| **48** | 0.4 ± 0.1 | 1.8 ± 0.7 | 2.2 ± 0.2 | 2.5 ± 0.5 | 1.1 ± 0.2 | 3.1 ± 0.6 | 1.2 ± 0.2 | 2.2 ± 0.2 | 2.5 ± 0.5 | 3.8 ± 0.8 |
| **49** | 0.4 ± 0.2 | 1.1 ± 0.3 | 1.0 ± 0.1 | 2.0 ± 0.7 | 0.7 ± 0.3 | 3.0 ± 0.5 | 1.1 ± 0.6 | 2.4 ± 0.6 | 1.8 ± 0.5 | 4.1 ± 0.3 |
| **50** | 0.4 ± 0.2 | 1.9 ± 0.6 | 1.2 ± 0.4 | 3.5 ± 0.8 | 0.5 ± 0.1 | 2.7 ± 0.1 | 1.1 ± 0.2 | 2.1 ± 0.5 | 1.5 ± 0.3 | 3.4 ± 0.2 |
| **51** | 1.3 ± 0.4 | 1.3 ± 0.5 | 2.3 ± 0.9 | 4.0 ± 0.9 | 3.3 ± 0.8 | 4.7 ± 0.8 | 2.0 ± 0.4 | 5.3 ± 0.9 | 3.4 ± 0.6 | 7.0 ± 0.4 |
| **52** | 0.8 ± 0.3 | 2.4 ± 0.5 | 6.4 ± 1.0 | 3.5 ± 0.7 | 3.2 ± 0.8 | 15.1 ± 2.1 | 3.2 ± 0.8 | 4.2 ± 0.7 | 3.3 ± 0.5 | 4.3 ± 0.6 |
| **53** | 0.4 ± 0.2 | 2.9 ± 0.5 | 3.2 ± 0.8 | 3.6 ± 0.2 | 1.7 ± 0.6 | 4.2 ± 0.8 | 2.3 ± 0.4 | 4.6 ± 0.7 | 6.0 ± 0.7 | 7.4 ± 0.5 |
| **54** | 3.0 ± 0.8 | 3.1 ± 0.3 | 1.6 ± 0.3 | 3.3 ± 0.6 | 1.6 ± 0.3 | 8.5 ± 1.5 | 2.4 ± 0.9 | 5.2 ± 0.9 | 6.1 ± 0.9 | 7.4 ± 0.3 |
| **55** | 2.1 ± 0.5 | 2.6 ± 0.9 | 1.0 ± 0.3 | 2.5 ± 0.4 | 0.7 ± 0.2 | 6.1 ± 1.2 | 1.3 ± 0.3 | 5.0 ± 0.8 | 1.6 ± 0.6 | 3.8 ± 0.7 |
| **56** | 2.3 ± 0.4 | 1.7 ± 0.2 | 1.0 ± 0.2 | 1.1 ± 0.2 | 2.9 ± 0.5 | 3.2 ± 0.2 | 3.6 ± 0.8 | 3.2 ± 0.5 | 4.8 ± 0.8 | 5.8 ± 0.9 |
| **57** | 0.9 ± 0.4 | 1.9 ± 0.1 | 3.7 ± 1.0 | 6.0 ± 1.0 | 1.7 ± 0.6 | 3.5 ± 1.0 | 1.7 ± 0.6 | 4.8 ± 0.8 | 2.8 ± 0.1 | 2.1 ± 0.3 |
| **58** | 0.8 ± 0.3 | 2.0 ± 0.5 | 1.8 ± 0.7 | 3.6 ± 0.8 | 1.5 ± 0.3 | 1.9 ± 0.6 | 1.7 ± 0.5 | 4.0 ± 0.8 | 1.8 ± 0.9 | 1.8 ± 0.5 |
| **59** | 0.3 ± 0.2 | 1.6 ± 0.7 | 0.5 ± 0.2 | 1.3 ± 0.9 | 0.4 ± 0.1 | 1.1 ± 0.2 | 0.4 ± 0.1 | 1.6 ± 0.5 | 1.2 ± 0.5 | 0.7 ± 0.2 |
| **60** | 0.2 ± 0.1 | 0.7 ± 0.2 | 0.3 ± 0.1 | 0.5 ± 0.1 | 0.7 ± 0.2 | 1.7 ± 0.3 | 0.4 ± 0.1 | 0.8 ± 0.1 | 0.7 ± 0.2 | 1.0 ± 0.2 |
| **61** | 0.2 ± 0.1 | 1.6 ± 0.8 | 0.4 ± 0.1 | 0.5 ± 0.2 | 1.2 ± 0.5 | 3.5 ± 0.8 | 0.6 ± 0.3 | 1.6 ± 0.2 | 1.7 ± 0.3 | 3.4 ± 0.6 |
| **62** | 0.2 ± 0.1 | 1.5 ± 0.6 | 1.2 ± 0.4 | 1.2 ± 0.3 | 0.5 ± 0.1 | 1.4 ± 0.4 | 0.6 ± 0.2 | 1.9 ± 0.6 | 3.0 ± 0.8 | 4.0 ± 0.8 |
| **63** | 0.7 ± 0.2 | 0.7 ± 0.2 | 0.6 ± 0.2 | 1.1 ± 0.2 | 0.8 ± 0.4 | 2.6 ± 0.3 | 0.8 ± 0.5 | 0.9 ± 0.3 | 1.2 ± 0.6 | 1.6 ± 0.5 |
| **64** | 0.6 ± 0.3 | 0.8 ± 0.4 | 0.9 ± 0.2 | 1.4 ± 0.6 | 0.3 ± 0.1 | 0.7 ± 0.1 | 0.5 ± 0.1 | 0.4 ± 0.2 | 2.1 ± 0.3 | 4.0 ± 0.6 |
| **65** | 0.7 ± 0.2 | 0.6 ± 0.2 | 1.0 ± 0.4 | 1.6 ± 0.2 | 0.7 ± 0.5 | 1.1 ± 0.5 | 0.3 ± 0.1 | 0.2 ± 0.1 | 2.2 ± 0.4 | 3.6 ± 0.3 |
| **66** | 1.4 ± 0.8 | 1.1 ± 0.2 | 0.3 ± 0.4 | 0.6 ± 0.2 | 0.7 ± 0.2 | 1.1 ± 0.3 | 0.5 ± 0.1 | 0.4 ± 0.1 | 8.5 ± 1.0 | 9.1 ± 0.9 |
| **67** | 1.2 ± 0.5 | 1.0 ± 0.3 | 1.3 ± 0.3 | 1.9 ± 0.7 | 0.9 ± 0.3 | 1.3 ± 0.3 | 0.6 ± 0.1 | 1.0 ± 0.2 | 3.9 ± 0.3 | 2.4 ± 0.9 |
| **68** | 0.6 ± 0.2 | 0.6 ± 0.1 | 1.6 ± 0.2 | 1.9 ± 0.6 | 0.6 ± 0.2 | 0.9 ± 0.2 | 0.7 ± 0.2 | 0.8 ± 0.2 | 1.8 ± 0.6 | 3.4 ± 0.5 |

[a] Each experiment was independently performed three times.
[b] H = Hypoxia: the percentage of oxygen is 3%.
[c] N = Normoxia: the percentage of oxygen is 20%.

**Table 2.** The hypoxic cytotoxicity ratio (normoxia/hypoxia) of TPZ derivatives **46–68**.

| Comp. | HCR[a, b] | | | | | Comp. | HCR[a, b] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | HL-60 | BEL-7402 | ECA-109 | MCF-7 | PC-3 | | HL-60 | BEL-7402 | ECA-109 | MCF-7 | PC-3 |
| **TPZ** | 1.1 | 1.8 | 6.6 | 1.7 | 3.8 | **57** | 2.2 | 1.6 | 2.0 | 2.8 | no |
| **46** | 1.6 | 2.2 | 5.4 | 2.1 | 4.4 | **58** | 2.4 | 2.0 | 1.3 | 2.4 | 1.0 |
| **47** | 2.8 | no | 1.7 | 1.3 | 1.9 | **59** | 5.1 | 2.4 | 2.7 | 3.84 | no |
| **48** | 4.1 | 1.1 | 2.8 | 1.9 | 1.5 | **60** | 4.0 | 1.6 | 2.6 | 2.1 | 1.4 |
| **49** | 2.5 | 2.0 | 4.1 | 2.1 | 2.3 | **61** | 6.4 | 1.0 | 2.9 | 2.7 | 2.0 |
| **50** | 4.2 | 2.8 | 5.1 | 1.9 | 2.3 | **62** | 6.9 | no | 2.8 | 3.0 | 1.3 |
| **51** | 1.0 | 1.74 | 1.4 | 2.7 | 2.1 | **63** | 1.0 | 1.8 | 3.2 | 1.1 | 1.4 |
| **52** | 2.9 | no | 4.7 | 1.3 | 1.3 | **64** | 1.4 | 1.6 | 2.2 | no | 1.9 |
| **53** | 7.9 | 1.1 | 2.5 | 2.0 | 1.2 | **65** | no | 1.5 | 1.5 | no | 1.6 |
| **54** | 1.01 | 2.07 | 5.31 | 2.19 | 1.21 | **66** | no | 1.68 | 1.60 | no | 1.07 |
| **55** | 1.21 | 2.54 | 8.77 | 3.79 | 2.46 | **67** | no | 1.44 | 1.47 | 1.61 | no |
| **56** | no | 1.07 | 1.12 | no | 1.21 | **68** | 1.09 | 1.17 | 1.45 | 1.20 | 1.89 |

[a] The HCR was the ratio of $IC_{50}$ values in normoxia and in hypoxia.
[b] Each value was the mean of three times of independent experiment.

zene ring or at the 3-position of TPZ could affect the cytotoxic activity by changing the electronic properties and the lipophilic properties of the entire molecule. When the group was an electron-adopting substituent on the benzene ring such as chlorine or a nitro group (e.g. **61**, **62**, and **64**), it showed lower $IC_{50}$ values than that of an electron-donating substituent such as a methyl or a methyloxy group. The side chains at the 3-position of TPZ could intensively influence the cytotoxic activity. When the side chain was an alkyl or an aromatic group, the compounds showed lower $IC_{50}$ values than TPZ (e.g. **48–50**). At the same time, all the tested compounds showed different activity toward various kinds of cancer cells and exhibited higher activity toward HL-60 cells and ECA-109 cells, and lower activity toward PC-3 cells.

In conclusion, all compounds showed higher activity in hypoxia than in normoxia against all the tested cancer cells as shown in Table 2. Compounds with electron-adopting substituents on the benzene ring showed higher cytotoxic activity but lower hypoxia selectivity. The compounds with a nitro group showed excellent activity but were avoid of hypoxia selectivity (e.g. **64–68**). The chloro compounds (e.g. **61, 62**) showed exciting results in the cytotoxic activity and hypoxia selectivity and would be likely candidates to be further modified for obtaining second-generation prodrugs with optimized micropharmacokinetic properties. The pharmacokinetic experiment on these compounds is currently under investigation in our laboratory.

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## Experimental

Melting points were recorded on a Büchi B-540 melting point apparatus and are uncorrected (Büchi Labortechnik, Flawil, Switzerland). The $^1$H-NMR spectra were recorded on a Bruker Avance DMX-400 NMR-spectrometer (400 MHz, 29°C; Bruker Bioscience, Billerica, MA, USA). Chemical shift are given in ppm relative to tetramethylsilane (TMS) as internal standard (0 ppm). Infrared (IR) spectra were performed on a Bruker VECTOR 22 spectrophotometer ($cm^{-1}$). Mass spectra were recorded on an Esquire-LC-00075 mass spectrometer (Bruker). Purifications were performed by chromatography over silica gel. All reagents were purchased from commercial suppliers and dried and purified when necessary.

### Chemistry

The compounds **13–16** were synthesized according to the known method [9].

#### *3-Hydroxy-7-nitro-1,2,4-benzotriazine-1-oxide **17***

3-Hydroxy-1,2,4-benzotriazine-1-oxide **13** was nitrified by the mixture of nitric acid and vitriol at 60°C for 2 h, and then the mixture was poured into ice water (200 mL), the precipitate was filtrated and dried to get a yellow solid **17**; yield 92.3%; m.p. 211–213°C; MS (ESI): 209 [M+H]; $^1$H-NMR (DMSO, 400 MHz) δ: 11.34 (s, 1H, OH), 8.69 (s, 1H, CH), 8.51 (d, 1H, CH, $J$ = 8.8 Hz), 7.45 (d, 1H, CH, $J$ = 9.2 Hz).

#### *General procedure for the synthesis of 3-Chloro-7-substituted-1,2,4-benzotriazine-1-oxides **18–22***

3-Hydroxy-1,2,4-benzotriazine-1-oxide **13** (0.1 mol) and phosphorus oxychloride (70 mL) were refluxed for 30 min. The mixture was evaporated under vacuum and the residue was poured on ice. The product was crystallized from methanol to yield the compound **18**. Compounds **19–22** were also prepared by the procedure described above.

 

### *3-Chloro-1,2,4-benzotriazine-1-oxide* ***18***

Yield 86.0%; m.p. 119 – 120°C (lit. [11]: m.p. 117 – 118°C); MS (ESI): 182 [M+H]; $^1$H-NMR ($CDCl_3$, 400 MHz) δ: 8.45 (d, 1H, CH, *J* = 8.8 Hz), 8.02 (d, 2H, CH, *J* = 4.0 Hz), 7.78 – 7.81 (m, 1H, CH); IR (KBr): 3079, 3038, 2218, 1979, 1859, 1664, 1608, 1569, 1488, 1451, 1412, 1357, 1323, 1267, 1210, 1180, 1124, 1085, 967, 767 $cm^{-1}$.

### *3-Chloro-7-methyl-1,2,4-benzotriazine-1-oxide* ***19***

Yield 80%; m.p. 180 – 181°C (lit. [13]: m.p. 177 – 179°C); MS (ESI): 196 [M+H]; $^1$H-NMR ($CDCl_3$, 400 MHz) δ: 8.20 (s, 1H, CH), 7.89 (d, 1H, CH, *J* = 8.4 Hz), 7.82 (d, 1H, CH, *J* = 8.4 Hz), 2.612 (s, 3H, $CH_3$).

### *3-Chloro-7-methoxyl-1,2,4-benzotriazine-1-oxide* ***20***

Yield 76%; m.p. 184 – 186°C (lit. [14]: m.p. 188.5 – 190.5°C); MS (ESI): 212 [M+H]; $^1$H-NMR ($CDCl_3$, 400 MHz) δ: 7.89 (d, 1H, CH, *J* = 8.8 Hz), 7.66 (s, 1H, CH), 7.62 (d, 1H, CH, *J* = 8.8 Hz), 4.01 (s, 3H, $OCH_3$).

### *3-Chloro-7-chloro-1,2,4-benzotriazine-1-oxide* ***21***

Yield 89%; m.p. 154 – 156°C (lit. [14]: m.p. 157 – 158.5°C); MS (ESI): 216 [M+H]; $^1$H-NMR ($CDCl_3$, 400 MHz) δ: 8.40 (s, 1H, CH), 7.92 – 7.98 (m, 2H, CH).

### *3-Chloro-7-nitro-1,2,4-benzotriazine-1-oxide* ***22***

Yield 84.4%; m.p. 163 – 168°C; MS (ESI): 227 [M+H]; $^1$H-NMR ($CDCl_3$, 400 MHz) δ: 8.98 (s, 1H), 8.45 (d, 1H, CH, *J* = 9.2 Hz), 8.13 (d, 1H, CH, *J* = 9.2 Hz).

TPZ was prepared by the known method as a red solid [11] m. p. 222 – 224°C (lit. [11]: m.p. 220°C); MS (ESI): 179 [M+1]; $^1$H-NMR ($CDCl_3$, 400 MHz) d: 8.31 (d, 2H, CH, *J* = 8.8 Hz), 7.86 (t, 1H, CH, *J* = 7.6 Hz), 7.53 (t, 1H, CH, *J* = 7.6 Hz), 6.15 (s, 2H, $NH_2$); IR (KBr): 3413, 3263, 3084, 1626, 1596, 1517, 1487, 1416, 1356, 1165, 1099, 858, 833, 777, 767, 728 $cm^{-1}$.

### *General procedure for the synthesis of 3-Alkylamino-7-substituted-1,2,4-benzotriazine-1,4-dioxides* ***46 – 68***

A mixture of 3-chloro-1,2,4-benzotriazine-1-oxide **18** (2.75 mmol) and ethylamine (2.75 mmol) in ethanol (10 mL) was stirred at room temperature for 12 h and then the precipitate was filtrated to yield the 3-ethylamino-1,2,4-benzotriazine-1-oxide **23**. Compound **23** (2.00 mmol) was oxidized by 30% hydrogen peroxide (10 mL) and acetic acid (20 mL) at 60°C for 24 h. The mixture was neutralized by saturated aqueous sodium bicarbonate solution and extracted with dichloromethane (3 × 100 mL), and then the combined organic fractions were dried over anhydrous sodium sulfate and evaporated under vacuum. The residue was chromatographed over silica gel and eluted with a gradient of (25% – 100%) EtOAc/petroleum to get 3-ethylamino-1,2,4-benzotriazine-1,4-dioxide **46**. Compounds **47 – 68** were also prepared by the procedure described above.

### *3-Ethylamino-1,2,4-benzotriazine-1,4-dioxide* ***46***

Yield 32.7%; m.p. 183 – 184°C; MS (ESI): 207 [M+H]; $^1$H-NMR ($CDCl_3$, 400 MHz) δ: 8.25 (d, 2H, CH, *J* = 8.8 Hz), 7.80 (t, 1H, CH, *J* = 7.6 Hz), 7.43 (t, 1H, CH, *J* = 8.0 Hz), 7.04 (s, 1H, NH), 3.55 – 3.62 (m, 2H, $CH_2$), 1.29 (t, 3H, $CH_3$, *J* = 7.6 Hz); IR (KBr): 3263, 2982, 1618, 1594, 1491, 1411, 1359, 1323, 1181, 1158, 1107, 1085, 765, 720 $cm^{-1}$.

### *3-(n-Hexylamino)-1,2,4-benzotriazine-1,4-dioxide* ***47***

Yield 21.9%; m.p. 153 – 155°C; MS (ESI): 263 [M+H]; $^1$H-NMR ($CDCl_3$, 400 MHz) δ: 8.26 (t, 1H, CH, *J* = 6.4 Hz), 8.15 (d, 2H, CH, *J* = 8.4 Hz), 8.07 (t, 1H, CH, *J* = 8.8 Hz), 7.87 (t, 1H, NH, *J* = 8.0 Hz), 1.18 – 1.22 (m, 10H, $CH_2$), 0.77~0.80 (m, 3H, $CH_3$); IR (KBr): 3255, 3108, 2918, 2849, 1597, 1493, 1412, 1358, 1176, 1108, 1078, 860, 763, 721 $cm^{-1}$.

### *3-Cyclohexylamino-1,2,4-benzotriazine-1,4-dioxide* ***48***

Yield 15.3%; m.p. 190 – 196°C; MS (ESI): 261 [M+H]; $^1$H-NMR (DMSO, 400 MHz) δ: 8.17 (d, 1H, CH, *J* = 8.0 Hz), 8.10 (d, 1H, CH, *J* = 8.0 Hz), 7.88 – 7.94 (m, 2H, CH), 7.53 (t, 1H, NH, *J* = 7.6 Hz), 3.69 – 3.75 (m, 1H, CH), 1.87 (d, 2H, $CH_2$, *J* = 12.8 Hz), 1.73 (d, 2H, $CH_2$, *J* = 11.8 Hz), 1.60 (d, 1H, $CH_2$, *J* = 13.2 Hz), 1.43~ 1.53 (m, 2H, $CH_2$), 1.26 – 1.37 (m, 2H, $CH_2$), 1.11 – 1.17 (m, 1H, $CH_2$); IR (KBr): 3246, 1739, 1619, 1493, 1414, 1357, 1227, 1184, 1089, 970, 865, 771, 725 $cm^{-1}$.

### *3-Phenylamino-1,2,4-benzotriazine-1,4-dioxide* ***49***

Yield 17.6%; m.p. 203 – 204°C; MS (ESI): 255 [M+H]; $^1$H-NMR ($CDCl_3$, 400 MHz) δ: 8.15 (d, 2H, CH, *J* = 8.4 Hz), 8.09 (d, 2H, CH, *J* = 8.4 Hz), 7.92 (d, 2H, CH, *J* = 8.0 Hz), 7.89 (t, 2H, CH, *J* = 6.8 Hz), 7.52 (t, 2H, CH, NH, *J* = 7.2 Hz); IR (KBr): 3309, 2928, 1646, 1590, 1491, 1366, 1174, 1105, 1025, 998, 839, 771, 725 $cm^{-1}$.

### *3-Benzylamino-1,2,4-benzotriazine-1,4-dioxide* ***50***

Yield 33.2%; m.p. 206 – 207°C; MS (ESI): 269 [M+H]; $^1$H-NMR ($CDCl_3$, 400 MHz) δ: 8.84 (t, 1H, CH, *J* = 6.4 Hz), 8.01 – 8.15 (m, 2H, CH), 7.89 (t, 1H, CH, *J* = 7.2 Hz), 7.51 (t, 1H, NH, *J* = 8.0 Hz), 7.34 (d, 2H, $CH_2$, *J* = 8.8 Hz), 7.27 (t, 2H, CH, *J* = 7.2 Hz), 7.20 (t, 1H, CH, *J* = 5.6 Hz), 4.58 (d, 2H, CH, *J* = 6.8 Hz); IR (KBr): 3387, 3012, 1603, 1574, 1498, 1417, 1356, 1206, 1188, 1103, 1085, 844, 726 $cm^{-1}$.

### *3-Ethylamino-7-methyl-1,2,4-benzotriazine-1,4-dioxide* ***51***

Yield 35.9%; m.p. 201 – 203°C; MS(ESI): 221 [M+H]; $^1$H-NMR ($CDCl_3$, 400 MHz): δ 8.19 (d, 1H, CH, *J* = 8.8 Hz), 8.13 (s, CH, 1H), 7.70 (d, 1H, CH, *J* = 8.8 Hz), 7.01 (s, 1H, NH), 3.63~3.66 (m, 2H, $CH_2$), 2.527 (s, 3H, $CH_3$), 1.340 – 1.377 (t, 3H, $CH_3$, *J* = 7.2 Hz); IR (KBr): 3254, 1608, 1507, 1414, 1379, 1354, 1330, 1160, 1091, 996, 894, 814, 777, 725 $cm^{-1}$.

### *3-(n-Hexylamino)-7-methyl-1,2,4-benzotriazine-1,4-dioxide* ***52***

Yield 28.7%; m.p. 162 – 170°C; MS (ESI): 277 [M+H]; $^1$H-NMR ($CDCl_3$, 400 MHz) δ: 8.18 (d, 1H, CH, *J* = 8.8 Hz), 8.12 (s, 1H, CH), 7.69 (d, 1H, CH, *J* = 8.8 Hz), 7.04 (s, 1H, NH), 3.55 – 3.61(m, 2H, $CH_2$), 2.52 (s, 3H, $CH_3$), 1.31 – 1.44 (m, 8H, $CH_2$), 0.84 – 0.91 (m, 3H, $CH_3$); IR (KBr): 3264, 2923, 2854, 1604, 1504, 1464, 1415, 1379, 1344, 1314, 1286, 1230, 1157, 1135,1110, 1083, 1014, 984, 897, 815, 786 $cm^{-1}$.

### *3-Cyclohexylamino-7-methyl-1,2,4-benzotriazine-1,4-dioxide* ***53***

Yield 38.6%; m.p. 201 – 203°C; MS (ESI): 275 [M+H]; $^1$H-NMR ($CDCl_3$, 400 MHz) δ: 8.17 (d, 1H, CH, *J* = 8.4 Hz), 8.11 (s, 1H, CH), 7.68 (d, 1H, CH, *J* = 8.4 Hz), 6.96 (s, 1H, NH), 3.94 – 3.97 (m, 1H, CH), 2.51 (s, 3H, $CH_3$), 2.07 – 2.10 (d, 2H, $CH_2$, *J* = 10.0 Hz), 1.65 – 1.68 (d, 2H, $CH_2$, *J* = 13.2 Hz), 1.36~1.45 (m, 4H, $CH_2$), 1.23 – 1.28

 

(m, 2H, $CH_2$); IR (KBr): 3250, 2936, 2855, 1661, 1584, 1501, 1414, 1375, 1341, 1291, 1232, 1254, 1112, 1087,1019, 960, 925, 892, 870, 847, 809, 774 $cm^{-1}$.

### *3-Phenylamino-7-methyl-1,2,4-benzotriazine-1,4-dioxide* ***54***

Yield 15.3%; m.p. 221–223°C; MS (ESI): 269 [M+H]; $^1$H-NMR ($CDCl_3$, 400 MHz) δ: 8.04 (d, 1H, CH, *J* = 8.8 Hz), 7.99 (s, 1H, CH), 7.92 (m, 5H, CH), 7.75 (d, 1H, CH, *J* = 8.8 Hz), 7.00 (s, 1H, NH), 3.31 (s, 3H, $CH_3$); IR (KBr): 3408, 3265, 1604, 1516, 1416, 1353, 1160, 1100, 1024, 900, 812, 770 $cm^{-1}$.

### *3-Benzylamino-7-methyl-1,2,4-benzotriazine-1,4-dioxide* ***55***

Yield 37.6%; m.p. 222–223°C; MS (ESI): 283 [M+H]; $^1$H-NMR (DMSO, 400 MHz) δ: 8.26 (d, 1H, CH, *J* = 8.8 Hz), 8.15 (s, 1H, NH), 7.75 (d, 2H, CH, *J* = 8.8 Hz), 7.25–7.26 (m, 5H, CH), 6.26 (s, 2H, $CH_2$), 2.55 (s, 3H, $CH_3$); IR (KBr): 3408, 3266, 1604, 1516, 1353, 1334, 1160, 1100, 1024, 900,812, 770 $cm^{-1}$.

### *3-Ethylamino-7-methyloxy-1,2,4-benzotriazine-1,4-dioxide* ***56***

Yield 23.5%; m.p. 205–207°C; MS (ESI): 237 [M+H]; $^1$H-NMR ($CDCl_3$, 400 MHz) δ: 8.19 (d, 1H, CH, *J* = 9.2 Hz), 7.57 (s, 1H, CH), 7.49 (d, 1H, CH, *J* = 9.2 Hz), 6.90 (s, 1H, NH), 3.94 (s, 3H, $OCH_3$), 3.57~3.64 (m, 2H, $CH_2$), 1.30 (t, 3H, $CH_3$, *J* = 6.8 Hz); IR (KBr): 3245, 1605, 1504, 1472, 1386, 1358, 1310, 1257, 1198, 1155, 1112,1081, 1008, 898,857, 826, 787 $cm^{-1}$.

### *3-Cyclohexylamino-7-methyloxy-1,2,4-benzotriazine-1,4-dioxide* ***57***

Yield 26.1%; m.p. 208–209°C; MS (ESI): 291 [M+H]; $^1$H-NMR ($CDCl_3$, 400 MHz) δ: 8.18 (d, 1H, CH, *J* = 9.2 Hz), 7.56 (s, 1H, CH), 7.47 (d, 1H, CH, *J* = 9.2 Hz), 6.82 (s, 1H, NH), 3.92 (s, 4H, CH, $OCH_3$), 2.08 (d, 2H, $CH_2$, *J* = 9.6 Hz), 1.77–1.80 (m, 2H, $CH_2$), 1.65–1.67 (d, 1H, $CH_2$, *J* = 9.6 Hz), 1.35–1.47 (m, 4H, $CH_2$), 1.24–1.32 (m, 1H, $CH_2$); IR (KBr): 3247, 2939, 1591, 1506, 1453, 1421, 1389, 1332, 1292, 1257, 1183, 1147, 1116, 1094, 1018, 964, 891,842, 816, 779 $cm^{-1}$.

### *3-Benzylamino-7-methyloxy-1,2,4-benzotriazine-1,4-dioxide* ***58***

Yield 24.8%; m.p. 210–214°C; MS (ESI): 299 [M+H]; $^1$H-NMR (DMSO, 400 MHz) δ: 8.11 (d, 1H, CH, *J* = 8.4 Hz), 8.04 (s, 1H, CH), 7.45 (m, 5H, CH), 7.37 (d, 1H, CH, *J* = 8.4 Hz), 6.78 (s, 1H, NH), 4.87 (s, 2H, $CH_2$), 3.78 (s, 3H, $OCH_3$); IR (KBr): 3423, 3352, 1646, 1535, 1473, 1415, 1397, 1358, 1275, 1204, 1168, 1121,1037, 845, 789 $cm^{-1}$.

### *3-Ethylamino-7-chloro-1,2,4-benzotriazine-1,4-dioxide* ***59***

Yield 39.5%; m.p. 205–209°C; MS (ESI): 241 [M+H]; $^1$H-NMR ($CDCl_3$, 400 MHz) δ: 8.33 (s, 1H, CH), 8.24 (d, 1H, CH, *J* = 9.2 Hz), 7.78 (d, 1H, CH, *J* = 9.2 Hz), 7.09 (s, 1H, NH), 3.60–3.67 (m, 2H, $CH_2$), 1.36 (t, 3H, $CH_3$, *J* = 7.2 Hz); IR (KBr): 3243, 2971, 2361, 2338, 1607, 1489, 1442, 1413, 1386, 1359, 1332, 1290, 1204, 1169, 1127, 1091, 1059, 993, 975, 873, 823, 806, 756 $cm^{-1}$.

### *3-(n-Hexylamino)-7-chloro-1,2,4-benzotriazine-1,4-dioxide* ***60***

Yield 31.3%; m.p. 181–183°C; MS (ESI): 297 [M+H]; $^1$H-NMR ($CDCl_3$, 400 MHz) δ: 8.34 (d, 1H, CH, *J* = 8.8 Hz), 8.26 (s, 1H, CH), 7.78 (d, 1H, CH, *J* = 8.8 Hz), 7.09 (s, 1H, NH), 3.56–3.61 (m, 2H, $CH_2$), 1.68~1.73 (m, 2H, $CH_2$), 1.40–1.44 (m, 2H, $CH_2$), 1.30–1.35 (m, 4H, $CH_2$), 0.68~0.91 (m, 3H, $CH_3$); IR (KBr): 3408, 3294, 3070, 1610, 1490, 1407, 1380, 1313, 1159, 1120, 1087, 913, 815, 755 $cm^{-1}$.

### *3-Cyclohexylamino-7-chloro-1,2,4-benzotriazine-1,4-dioxide* ***61***

Yield 45.8%; m.p. 191–192°C; MS (ESI): 295 [M+H]; $^1$H-NMR ($CDCl_3$, 400 MHz) δ: 8.33 (s, 1H, CH), 8.24 (d, 1H, CH, *J* = 8.8 Hz), 7.78 (d, 1H, CH, *J* = 8.8 Hz), 7.01 (s, 1H, NH), 3.94–3.98 (m, 1H, CH), 2.05–2.10 (m, 2H, $CH_2$), 1.79~1.83 (m, 2H, $CH_2$), 1.86–1.90 (m, 1H, $CH_2$), 1.35~1.49 (m, 4H, $CH_2$), 1.23–1.30 (m, 1H, $CH_2$); IR (KBr): 3290, 3090, 3068, 2927, 1592, 1489, 1462, 1445, 1405, 1382, 1371, 1334, 1298, 1272, 1193, 1162, 1120, 1107, 1082, 961, 912, 884, 845, 817, 756 $cm^{-1}$.

### *3-Phenylamino-7-chloro-1,2,4-benzotriazine-1,4-dioxide* ***62***

Yield 27.3%; m.p. 295°C; MS (ESI): 289 [M+H]; $^1$H-NMR ($CDCl_3$, 400 MHz) δ: 8.36 (s, 1H, CH), 8.35 (d, 1H, CH, *J* = 9.0 Hz), 8.33 (d, 1H, CH, *J* = 9.0 Hz), 7.83–7.86 (m, 5H, CH), 7.01 (s, 1H, NH); IR (KBr): 3408, 3276, 3064, 1621, 1600, 1523, 1487, 1403, 1381, 1358, 1164, 1097, 1024, 915, 876, 815, 751 $cm^{-1}$.

### *3-Benzylamino-7-chloro-1,2,4-benzotriazine-1,4-dioxide* ***63***

Yield 42.2%; m.p. 275–276°C; MS (ESI): 303 [M+H]; $^1$H-NMR($CDCl_3$, 400 MHz) δ: 8.34 (s, 1H, CH), 8.26 (d, 1H, CH, *J* = 9.2 Hz), 7.94 (d, 1H, CH, *J* = 9.2 Hz), 7.31–7.44 (m, 5H, CH), 5.30 (s, 1H, NH), 7.77 (s, 2H, $CH_2$); IR (KBr): 3411, 3283, 3091, 3068, 1600, 1526, 1489, 1407, 1379, 1358, 1325, 1261, 1163, 1123, 1095, 1024, 950, 915, 877, 816, 753 $cm^{-1}$.

### *3-Ethylamino-7-nitro-1,2,4-benzotriazine-1,4-dioxide* ***64***

Yield 33.6%; m.p. 203–205°C; MS (ESI): 252 [M+H]; $^1$H-NMR ($CDCl_3$, 400 MHz) δ: 8.93 (s, 1H, CH), 8.60 (d, 1H, CH, *J* = 9.6 Hz), 8.42 (d, 1H, CH, *J* = 9.6 Hz), 7.32 (s, 1H, NH), 3.70–3.70 (m, 2H, $CH_2$), 1.40 (t, 3H, $CH_3$, *J* = 6.8 Hz); IR (KBr): 3233, 3101, 1634, 1601, 1530, 1497, 1428, 1392, 1331, 1236, 1161, 1132, 1093, 995, 920, 894, 830, 802, 745 $cm^{-1}$.

### *3-(n-Hexylamino)-7-nitro-1,2,4-benzotriazine-1,4-dioxide* ***65***

Yield 25.3%; m.p. 201–204°C; MS (ESI): 308 [M+H]; $^1$H-NMR ($CDCl_3$, 400 MHz) δ: 8.91 (s, 1H, CH), 8.86 (d, 1H, CH, *J* = 10.0 Hz), 8.55 (d, 1H, CH, *J* = 11.6 Hz), 8.24 (s, 1H, NH), 3.30~3.42 (m, 2H, $CH_2$), 3.31–3.38 (m, 4H, $CH_2$), 1.62–1.67 (m, 4H, $CH_2$), 0.92 (t, 3H, $CH_3$, *J* = 7.2 Hz); IR (KBr): 3251, 3080, 1633, 1602, 1529, 1498, 1471, 1432, 1392, 1330, 1296, 1157, 1134, 1107, 1082, 1056, 981, 920, 831, 750 $cm^{-1}$.

 

### *3-Cyclohexylamino-7-nitro-1,2,4-benzotriazine-1,4-dioxide* ***66***

Yield 39.4%; m.p. 178 – 183°C; MS (ESI): 306 [M+H]; $^1$H-NMR (DMSO, 400 MHz) δ: 8.88 (s, 1H, CH), 8.54 (d, 1H, CH, *J* = 9.8 Hz), 8.25 (d, 1H, CH, *J* = 9.8 Hz), 6.89 (s, 1H, NH), 3.81 (s, 1H, CH), 3.31~3.48 (m, 2H, $CH_2$), 1.85 – 1.88 (m, 2H, $CH_2$), 1.74 – 1.78 (m, 2H, $CH_2$), 1.30 – 1.36 (m, 4H, $CH_2$); IR (KBr): 3311, 3277, 3058, 2940, 2856, 1592, 1530, 1494, 1465, 1418, 1393, 1372, 1336, 1292, 1159, 1135, 1104, 1084, 1022, 962, 942, 924, 891, 841, 749 $cm^{-1}$.

### *3-Phenylamino-7-nitro-1,2,4-benzotriazine-1,4-dioxide* ***67***

Yield 19.1%; m.p. 257 – 261°C; MS (ESI): 300 [M+H]; $^1$H-NMR(DMSO, 400 MHz) δ: 8.85 (s, 1H, CH), 8.57 (d, 1H, CH, *J* = 11.6 Hz), 8.30 (d, 1H, CH, *J* = 11.6 Hz), 7.59 – 7.87 (m, 5H, CH), 7.22 (s, 1H, NH); IR (KBr): 3411, 3278, 3106, 3062, 1866, 1744, 1632, 1604, 1535, 1510, 1489, 1472, 1413, 1394, 1361, 1341, 1265, 1166, 1099, 938, 913, 854, 815, 747 $cm^{-1}$.

### *3-Benzylamino-7-nitro-1,2,4-benzotriazine-1,4-dioxide* ***68***

Yield 31.9%; m.p. 276 – 281°C; MS (ESI): 314 [M+H]; $^1$H-NMR (DMSO, 400 MHz) δ: 8.83 (s, 1H, CH), 8.64 (d, 1H, CH, *J* = 11.6 Hz), 8.27 (d, 1H, CH, *J* = 11.6 Hz), 7.43 – 7.75 (m, 5H, CH), 7.10 (s, 1H, NH), 4.69 (s, 2H, $CH_2$); IR (KBr): 3404, 3274, 3110, 3055, 2921, 2851, 1632, 1602, 1536, 1489, 1471, 1412, 1385, 1361, 1338, 1261, 1165, 1099, 1026, 941, 912, 840, 813, 747 $cm^{-1}$.

### Cell culture and cytotoxicity assay

All cancer cells, promyelocytic leukemia HL-60, androgen-independent prostate tumor PC-3, hepatocellular carcinoma BEL-7402, human esophagus tumor ECA-109, and human breast cancer MCF-7, were cultured in RPMI-1640 medium with heat-inactivated 10% fetal bovine serum in hypoxic atmosphere with 3% $O_2$, 5% $CO_2$ and in normoxic atmosphere with 20% $O_2$, 5% $CO_2$ at 37°C. All the compounds were dissolved in DMSO at the concentrations 1.0 mg/mL and were then diluted to the appropriate concentrations. Cells were seeded in 96-well microtiter plates (4000 cells/well). After 24-hour incubation in appropriate medium, cells were treated with various concentrations (0, 0.2, 1.0, 5.0, 25.0, 50.0 μM) of all tested compounds, and incubated in normoxia and in hypoxia for 72 h, respectively. Afterwards, 10 μL of stock 3-[4,5-dimethylthia-zol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma) solution was added to each well (final: 0.25 mg/mL) for another 4 h of incubation. After these 4 h, 100 μL of DMSO was added to each well and optical density (OD) was read at 570 nm. The $IC_{50}$ values were calculated using the PrismPad computer program (GraphPad Software, Inc., San Diego, CA, USA) and were defined as concentration of drug causing 50% inhibition in absorbance compared with control (vehicle) cells. All of the experiments were performed in triplicate and the $IC_{50}$ values were derived from the mean OD values of the triplicate tests.

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