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Synthesis of (1,3,4-thiadiazol-2-yl)-acrylamide derivatives as potential antitumor agents against acute leukemia cells

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ABSTRACT

A lead compound with the (1,3,4-thiadiazol-2-yl)-acrylamide scaffold was discovered to have significant cytotoxicity on several tumor cell lines in an in-house cell-based screening. A total of 60 derivative compounds were then synthesized and tested in a CCK-8 cell viability assay. Some of them exhibited improved cytotoxic activities. The most potent compounds had IC_{50} values of 1–5 μM on two acute leukemia tumor cell lines, i.e. RS4;11 and HL-60. Flow cytometry analysis of several active compounds and detection of caspase activation indicated that they induced caspase-dependent apoptosis. It was also encouraging to observe that these compounds did not have obvious cytotoxicity on normal cells, i.e. $IC_{50} > 50$ μM on HEK-293T cells. Although the molecular targets of this class of compound are yet to be revealed, our current results suggest that this class of compound represents a new possibility for developing drug candidates against acute leukemia.

With the advances in molecular biology and biochemistry, target-based drug discovery, also known as 'rational drug discovery', is prevalent in the development of new drugs since this century.[1,2] However, many kinds of research indicate that all the major diseases are multifactorial and involve complex gene or target networks. This may explain the dilemma faced by the target-based drug discovery.[3] Thus, the traditional cell-based screening remains essential for discovering compounds with desired potencies, as a complementary approach to target-based screening.

In our efforts on the development of anti-tumor agents, we screen all compounds available at the cellular level. These compounds are purchased from a commercial resource or synthesized by ourselves. In our cell-based screening protocol, each compound is tested at a single concentration of 20 μM in a CCK-8 cell viability assay on five selected tumor cell lines,[4] including A549 (human alveolar epithelial cell), HeLa (human cervical tumor cell), MBA-MD-231 (human breast tumor cell), RS4;11 (human acute lymphoblastic leukemia tumor cell) and HL-60 (human protomyeloid leukemia tumor cell). For example, compound **1** (Fig. 1) was discovered in our screening, which exhibited significant cytotoxicity especially on two types of acute leukemia tumor cell lines, RS4;11 and HL-60. The inhibition ratio at this concentration was over 50% on RS4;11 and HL-60 cells. The IC_{50} value on HL-60 cells was 12 μM. Current anti-tumor drugs on the market are mainly used for the therapies of common cancers with high incidences, such as liver cancer, lung cancer, and stomach cancer. While for some rare malignant tumors, like cervical cancer, acute leukemia, etc., there are still relatively few types of small-molecule drugs available, and the treatment is usually limited.[5,6] Therefore, compound **1** could be a good starting point for developing effective candidate drug against acute leukemia.

In terms of chemical structure, compound **1** contains two distinct moieties: a 1,3,4-thiadiazol moiety and a sulfonic acid phenolate moiety. Our literature survey indicates that some known compounds containing the 1,3,4-thiadiazol moiety are reported to have anti-tumor effects (Fig. 2). For example, Kumar et al reported a class of 2-arylamino-5-aryl-1,3,4-thiadiazoles (compound **2**), which showed cytotoxicity on several cancer cell lines, such as prostate, pancreatic and breast tumor cells.[7] Compound **4**, i.e. N-((5-(substituted methylene amino)-1,3,4-thiadiazol-2-yl)-methyl) benzamide, also had anti-tumor

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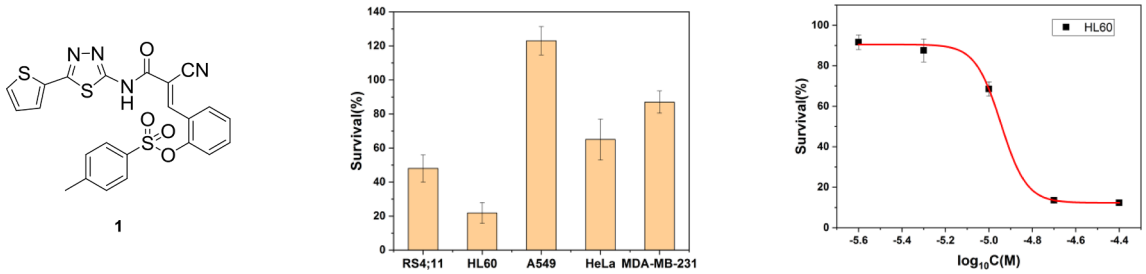


Fig. 1. Chemical structure of compound **1** (left) and its cytotoxicity on five tumor cell lines (middle). Its IC_{50} value on HL-60 cells is 12 μM (right).

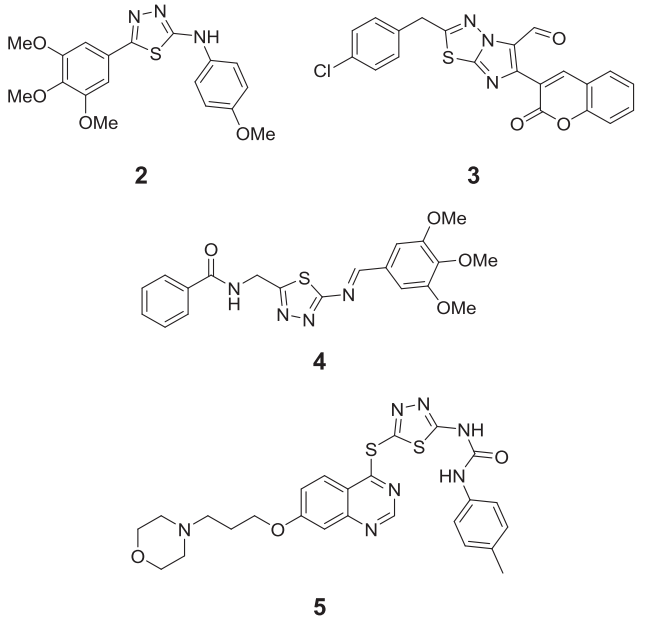
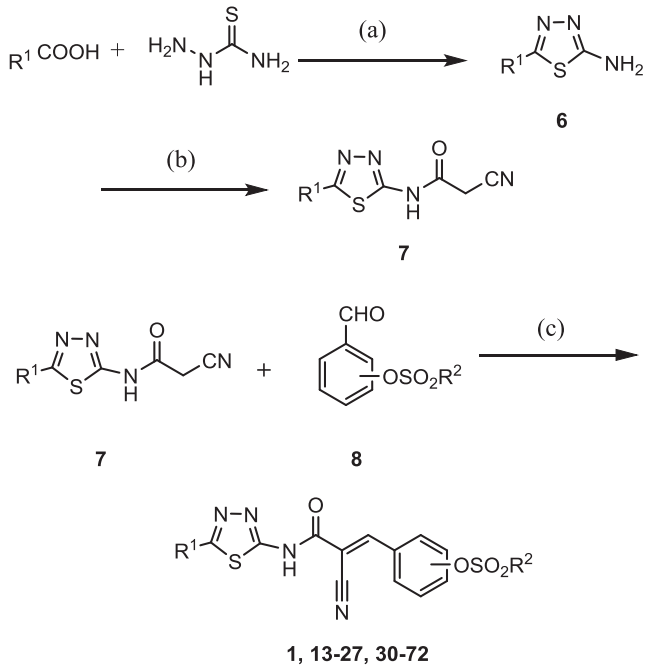


Fig. 2. Examples of anti-tumor compounds which contain the 1,3,4-thiodiazole scaffold.



Scheme 1. Synthetic route for obtaining compounds **1**, **13–27**, and **30–72** reported here. Reagents and conditions: (a) $POCl_3$, 70 °C; (b) i. EtO_2CCH_2CN, ii. NaOMe/MeOH, reflux, 5 h; (c) i. NH_4OAc, AcOH ii. tulene, reflux, 8 h.

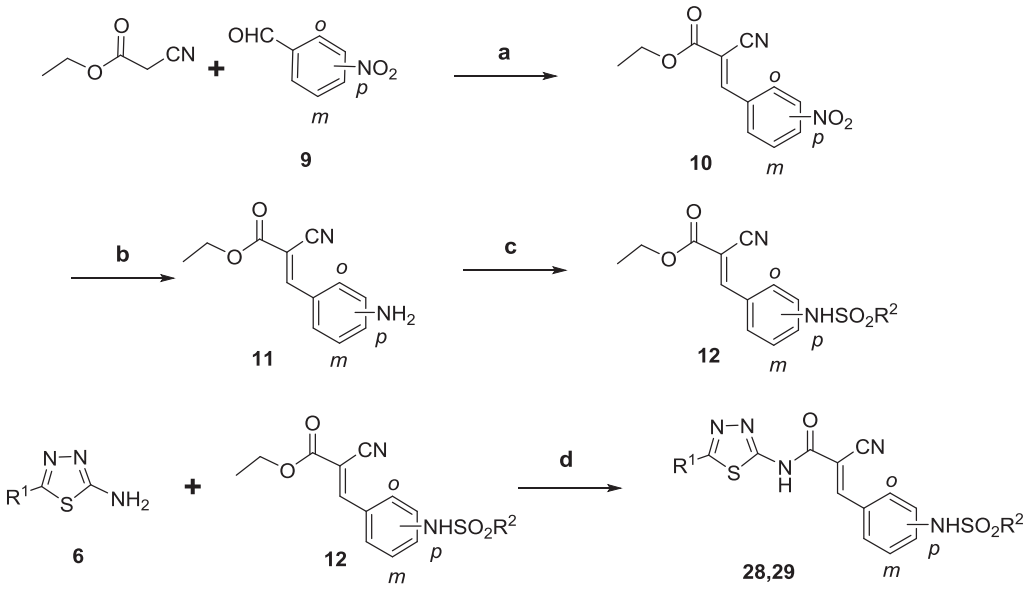
effect on several types of tumor cells.[8] The inhibitory activity of 1,3,4-thiadiazole derivatives on leukemia cells has also been reported, such as compound **3** and **5**.[9,10] Therefore, we consider 1,3,4-thiadiazole moiety as a valuable scaffold for developing new anti-tumor compounds. The sulfonic acid group usually exists in the salt form in a drug molecule, which leads to good solubility and other pharmacological properties.[11] However, the downside is that the sulfonic acid group can easily form a sulfonate with alkyl alcohol, such as methyl methanesulfonate, methyl benzenesulfonate and etc. The sulfonate can directly or indirectly react with DNA to transfer the alkyl group and cause damage[12]. Nevertheless, compound **1** contains a phenol ester, which is in principle more stable than alcohol esters and thus may avoid the potential genotoxicity. Thus, we believe that it is possible to develop a new class of anti-tumor compounds against acute leukemia based on the scaffold of compound **1**.

In this work, a total of 60 derivatives of compound **1** were synthesized with the methods outlined in Scheme 1 and 2. Using the conditions described in literature,[13,14] carboxylic acid and hydrazinecarbothioamide were mixed to produce the intermediate 5-substituted-1,3,4-thiadiazol-2-amine (**6**) via a condensation reaction. Then, under basic condition (NaOMe/MeOH), compound **6** was reacted with ethyl cyanoacetate to obtain 2-cyano-N-(5-substituted-1,3,4-thiadiazole-2-)acetamide (**7**). The aromatic aldehyde intermediate **8** was prepared by hydroxybenzaldehyde and the corresponding substituted benzenesulfonyl chloride in the mixture of DMAP/TEA/CH_2Cl_2.[15] At the last step, **7** and **8** were refluxed in the solution of NH_4OAc/HOAc/PhMe[16] for 8 h to produce compounds **1**, **13–27**, and **30–72** with overall yields of 20–80%.

To obtain the benzenesulfonamide derivatives, we adopted a modified synthetic route as illustrated in Scheme 2. Ethyl cyanoacetate was first condensed with nitrobenzaldehyde to obtain **10**, which was subsequently reduced to its amine **11** with $SnCl_2$/MeOH.[17] **11** was then reacted with a sulfonyl chloride to produce the intermediate **12**. Under the catalysis of the alkaline reagent, **6** and **12** underwent transamination at basic condition (CH_3ONa/CH_3OH) to produce the final targets **28** and **29**.

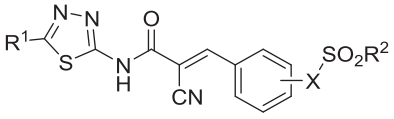
All of the synthesized compounds were then tested at a single concentration of 20 μM on RS4;11 and HL-60 cells. The HEK293T cell line was used as a model of normal cells in our work. The CCK-8 cell viability assay was used to determine the inhibition ratio of the tested compounds on tumor cell growth. The results obtained in this assay are summarized in Table 1. One can see that when the sulfonate or sulfonamide group is at the ortho position on the phenyl ring, the corresponding compounds with an electron donating group as R^2 achieve good inhibitory activities against two tumor cells. In contrast, electron withdrawing groups as R^2 reduce the inhibitory activities of the corresponding compounds, such as **14**, **16**, and **17**. Their inhibition ratios on two tumor cells are lower than 50%. A similar trend is observed among the compounds where the sulfonate or sulfonamide group are at the meta position on the phenyl ring.

The compounds with inhibition ratio over 50% in the initial screening were selected to be further tested at multiple concentrations to derive their concentrations at half inhibition (IC_{50}). In this assay,



Scheme 2. Synthetic route for obtaining compounds **28** and **29**. Reagents and conditions: (a) i. AcOH/$AcONH_4$, ii. PhMe, reflux, 5 h; (b) i. $SnCl_2{\cdot}2H_2O$ (5 equiv.), ii. EtOH/HCl; (c) i. CH_2Cl_2, pyridine, rt, ii. RSO_2Cl; (d) CH_3ONa/CH_3OH, reflux, 8 h.

Table 1
Chemical structures of compounds **1** and **13–72** and their cytotoxicity on three cell lines.



ID	R^1	R^2	Location of substituents on phenyl group	X	Inhibition ratio[a] (%) at 20 μM		
					RS4;11	HL-60	HEK293T
1	Me	4-Me-Ph	ortho	O	33	78	35
13	thiophene	4-CF_3-Ph	ortho	O	58	55	57
14	thiophene	4-Cl-Ph	ortho	O	22	17	46
15	thiophene	4-OMe-Ph	ortho	O	76	67	40
16	thiophene	4-NO_2-Ph	ortho	O	40	23	33
17	thiophene	4-OCF_3-Ph	ortho	O	39	48	42
18	2-indol	4-OCF_3-Ph	ortho	O	26	59	53
19	Me	Me	meta	O	33	20	20
20	Me	4-Me-Ph	meta	O	28	71	27
21	Ph	4-Me-Ph	meta	O	35	63	52
22	4-OMe-Ph	4-Me-Ph	meta	O	79	73	N.A.
23	2-Cl-Ph	4-Me-Ph	meta	O	44	67	55
24	2-OMe-Ph	4-Me-Ph	meta	O	41	73	N.A.[b]
25	thiophene	4-Me-Ph	meta	O	25	62	43
26	thiophene	4-NHAc-Ph	meta	O	33	71	20
27	thiophene	thiophene	meta	O	N.A.	30	50
28	thiophene	4-F-Ph	meta	NH	22	N.A.	29
29	thiophene	4-OMe-Ph	meta	NH	40	77	21
30	thiophene	Me	para	O	13	N.A.	19
31	4-OMe-Bn	4-Me-Ph	ortho	O	70	25	21
32	4-OMe-Bn	4-OMe-Ph	ortho	O	78	100	30
33	4-OMe-Bn	4-NO_2-Ph	ortho	O	70	51	17
34	4-OMe-Bn	2-NO_2-Ph	ortho	O	68	25	18
35	4-OMe-Bn	4-Cl-Ph	ortho	O	79	20	34
36	4-OMe-Bn	4-F-Ph	ortho	O	80	25	6
37	1-methylnaphthyl	4-Me-Ph	ortho	O	73	19	23
38	1-methylnaphthyl	4-OMe-Ph	ortho	O	78	98	29
39	1-methylnaphthyl	4-NO_2-Ph	ortho	O	58	18	25
40	1-methylnaphthyl	2-NO_2-Ph	ortho	O	66	23	N.A.
41	1-methylnaphthyl	4-Cl-Ph	ortho	O	68	15	16
42	1-methylnaphthyl	4-F-Ph	ortho	O	68	12	15
43	Bn	4-Me-Ph	ortho	O	69	16	15

(*continued on next page*)

Table 1 (*continued*)

ID	R^1	R^2	Location of substituents on phenyl group	X	Inhibition ratio[a] (%) at 20 μM		
					RS4;11	HL-60	HEK293T
44	Bn	4-OMe-Ph	ortho	O	78	100	23
45	Bn	4-NO_2-Ph	ortho	O	57	20	N.A.
46	Bn	2-NO_2-Ph	ortho	O	71	12	10
47	Bn	4-Cl-Ph	ortho	O	73	26	25
48	Bn	4-F-Ph	ortho	O	69	22	6
49	4-F-Bn	4-Me-Ph	ortho	O	72	N.A.	16
50	4-F-Bn	4-OMe-Ph	ortho	O	76	100	20
51	4-F-Bn	4-NO_2-Ph	ortho	O	55	18	8
52	4-F-Bn	2-NO_2-Ph	ortho	O	61	35	6
53	4-F-Bn	4-Cl-Ph	ortho	O	68	20	6
54	4-F-Bn	4-F-Ph	ortho	O	59	13	N.A.
55	3-F-Bn	4-Me-Ph	ortho	O	56	11	9
56	3-F-Bn	4-OMe-Ph	ortho	O	74	100	29
57	3-F-Bn	4-NO_2-Ph	ortho	O	65	18	N.A.
58	3-F-Bn	2-NO_2-Ph	ortho	O	68	23	N.A.
59	3-F-Ph	4-Cl-Ph	ortho	O	71	42	29
60	3-F-Bn	4-F-Ph	ortho	O	72	22	17
61	4-Cl-Bn	4-Me-Ph	ortho	O	69	15	20
62	4-Cl-Bn	4-OMe-Ph	ortho	O	77	99	26
63	4-Cl-Bn	4-NO_2-Ph	ortho	O	66	34	19
64	4-Cl-Bn	2-NO_2-Ph	ortho	O	65	19	20
65	4-Cl-Bn	4-Cl-Ph	ortho	O	64	15	14
66	4-Cl-Bn	4-F-Ph	ortho	O	64	20	14
67	4-Me-Bn	4-Me-Ph	ortho	O	66	17	22
68	4-Me-Bn	4-OMe-Ph	ortho	O	79	100	28
69	4-Me-Bn	4-NO_2-Ph	ortho	O	64	17	9
70	4-Me-Bn	2-NO_2-Ph	ortho	O	N.A.	33	6
71	4-Me-Bn	4-Cl-Ph	ortho	O	72	38	8
72	4-Me-Bn	4-F-Ph	ortho	O	73	11	11
ABT-199[c]					95	98	N.A.

[a] Cell growth inhibition ratio was measured after 48-hour treatment.
[b] No significant inhibitory activity observed at the test concentration.
[c] ABT-199 was tested at the concentration of 1 μM.

Table 2
Cytotoxicity profiling of the most potent compounds.

Compd	Cytotoxicity (IC_{50}, μM)[a]		
	RS4;11	HL-60	HEK-293T
1	> 20	11.47 ± 0.92	> 20
13	10.54 ± 2.01	17.46 ± 1.60	12.31 ± 2.45
15	4.75 ± 0.12	5.23 ± 0.85	> 20
18	> 20	11.06 ± 0.83	14.38 ± 1.46
20	> 20	5.78 ± 0.74	> 50
25	> 20	8.75 ± 2.38	> 20
26	> 20	7.94 ± 0.92	> 20
29	> 20	8.37 ± 0.61	> 20
32	0.84 ± 0.05	3.12 ± 0.05	> 50
33	3.67 ± 0.25	10.90 ± 0.42	> 50
35	4.02 ± 0.20	11.42 ± 0.29	> 50
38	1.36 ± 0.06	4.70 ± 0.09	> 50
44	1.06 ± 0.07	3.17 ± 0.18	> 50
50	1.21 ± 0.04	4.05 ± 0.62	> 50
56	1.21 ± 0.11	3.88 ± 0.69	> 50
62	1.25 ± 0.02	4.36 ± 0.43	> 50
68	1.01 ± 0.09	4.08 ± 0.53	> 50

[a] Each compound was tested in triplicate; the data are presented as the mean ± SD.

venetoclax (ABT-199), which is a potent apoptosis inducer and approved by FDA for treating adults with chronic lymphocytic leukemia (CLL) or small lymphocytic lymphoma (SLL), and treating adults with newly-diagnosed acute myeloid leukemia (AML) in combination therapies,[18,19] was used as the positive control compound. The IC_{50} values of all active compounds (on one or both tumor cell lines) are summarized in Table 2. Here, compounds **32**, **38**, **44**, **50**, **56**, **62** and **68** exhibit the most potent inhibitory activities on both RS4;11 and HL-60 cell lines (IC_{50} < 5 μM). Notably, all these compounds have 4-methoxyphenyl as the R^2 group. If this group is replaced by other groups, such as 4-nitrophenyl and 4-chlorophenyl, their inhibitory activities will decrease by 3–20 folds (Table S1 in Supporting Information). It is also encouraging to observe that compounds **32**, **38**, **44**, **50**, **56**, **62** and **68** are generally less toxic on HEK-293 T cells (IC_{50} > 50 μM), demonstrating the desired selectivity between tumor cells and normal cells.

In order to explore their mechanism in inducing apoptosis, we chose to characterize mitochondrial potential reduction, transfer of phosphatidylserine (PS), and change of plasma membrane permeability caused by these active compounds through flow cytometry analysis. Compounds **32, 50** and **62** were selected for flow cytometry analysis with Mitodamage staining, which could detect the above three events in apoptosis. After the treatment by the selected compounds at 5 μM, 10 μM, 20 μM, and 40 μM for 48 h, RS4;11 cells were stained with 7-AAD, annexin V, and MitoSense Red and then were analyzed. Here, Annexin V and 7-AAD were used to detect the state of PS and the plasma membrane permeability of cells, respectively. Combinations of 7-AAD and annexin V were used to distinguish cells in early apoptosis (i.e. annexin V (+) and 7-ADD (−)) and late apoptosis (i.e. annexin V (+) and 7-ADD (+)), corresponding to the bottom right and upper right sections in Fig. 3. For the cells treated by DMSO, about 5% of them were observed in early and late apoptosis. After the test compound was added, the ratio of apoptotic cells increased with the compound concentration (see Fig. 3 and Fig. S1 in Supporting Information). When the cells were treated by these compounds at 40 μM, the ratio of apoptotic cells reached 78%, 68%, and 81% for compounds **32**, **50** and **62**,

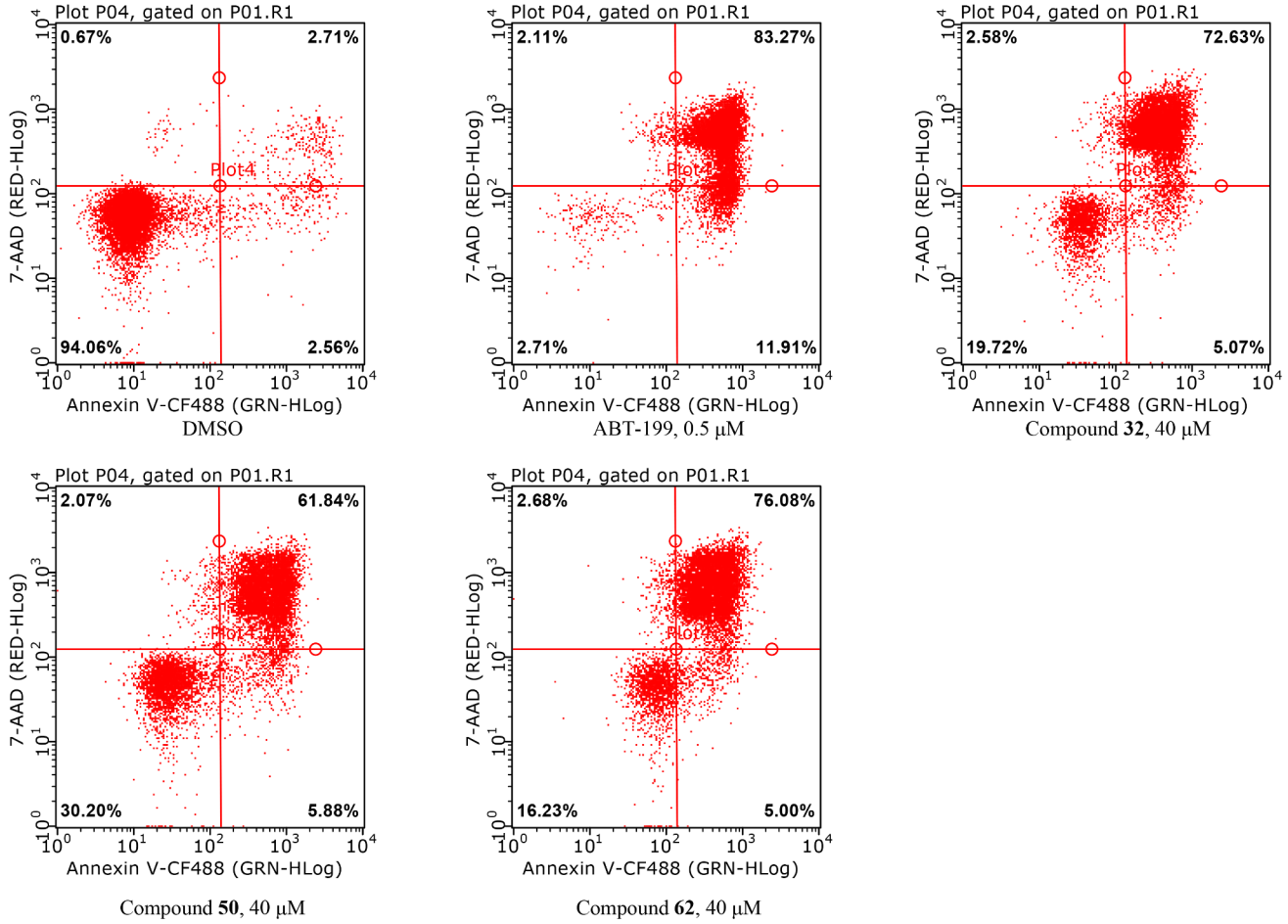
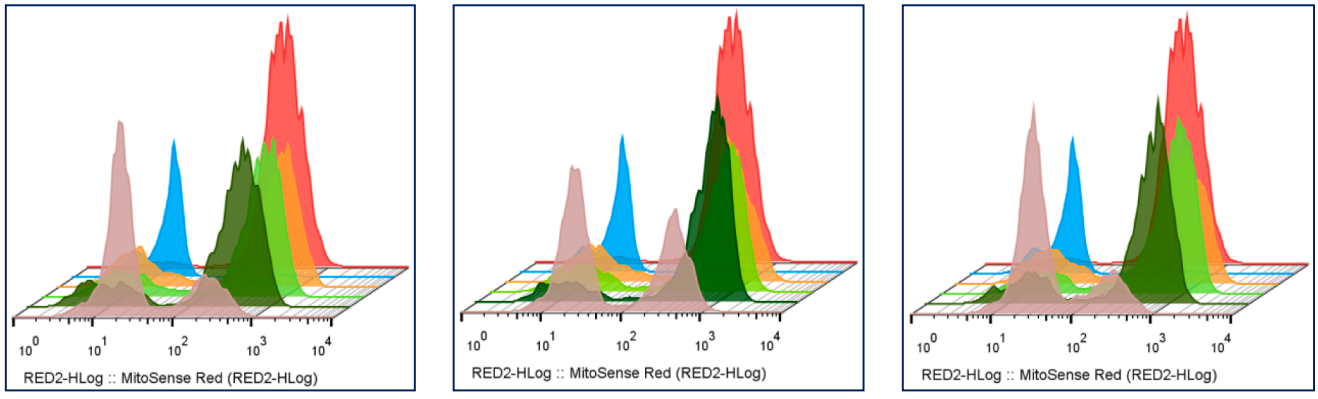


Fig. 3. Results of flow cytometric analysis with Annexin V and 7-AAD on RS4;11 cells for three selected compounds. Cells were treated for 48 h. Cells with the treatment of DMSO and ABT-199 were used as the negative and the positive controls respectively in this assay.



Compound 32 **Compound 50** **Compound 62**

Fig. 4. Results of flow cytometric analysis with MitoSense Red on RS4;11 cells for three selected compounds. Cells were treated at 5 μM (orange), 10 μM (light green), 20 μM (dark green) and 40 μM (skin pink) for 48 h. Cells with the treatment of DMSO (red) and ABT-199 (blue) were used as the negative and the positive controls respectively in this assay.

respectively. The MitoSense Red was used to detect the changes in mitochondrial membrane potential. Uninduced cells with intact mitochondrial membrane are associated with high Red2 fluorescence; while cells with impaired mitochondrial membrane are associated with low Red2 fluorescence. As one can see in Fig. 4, Red2 fluorescence shifted significantly to the left when the concentration of the compound under test reached 40 μM, indicating an obvious damage to mitochondrial membrane. This is consistent with the results from 7-AAD/annexin V staining, indicating that these compounds under test are capable of inducing cell apoptosis.

Poly-ADP-ribose polymerase protein (PARP) is a major substrate of activated caspases. Cleavage of PARP is a critical indicator of cell apoptosis. In our work, the three compounds that exhibited significant pro-apoptotic signals in flow cytometry analysis, i.e. **32**, **50** and **62**,

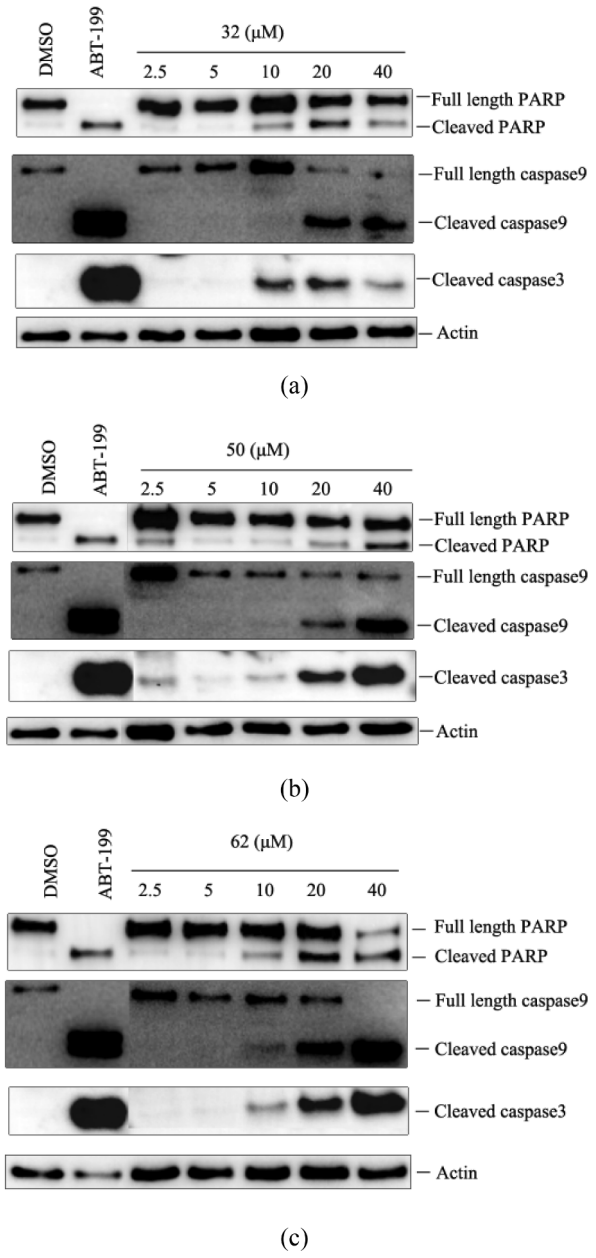


Fig. 5. Activation of caspase-3, caspase-9 and PARP by three selected compounds. RS4;11 cells were treated by these compounds at multiple concentrations for 48 h. Expression levels of caspase-3, caspase-9, and PARP were examined by western blot.

were further tested in the caspase activation assay. Again, DMSO and ABT-199 were used here as the negative and positive controls, respectively. Our results showed that compared to the negative control, the full-length PARP (116 kDa) was degraded and the cleaved PARP (89 kDa) was increased after treatment with the three given compounds (Fig. 5). After the compound concentration was increased from 2.5 μM to 40 μM, the cleaved PARP, cleaved caspase-3 and cleaved caspase-9 were increased generally in a dose-dependent manner. This observation again confirms the results of our flow cytometry analysis that these compounds are able to induce the caspase-dependent apoptosis.

In conclusion, we have developed a class of compounds with the (1,3,4-thiadiazol-2-yl)-acrylamide scaffold, which exhibited significant cytotoxicity on two acute leukemia cells, i.e. RS4;11 and HL-60. The most potent compounds have IC_{50} values lower than 5 μM. Flow cytometry analysis and cleaved caspase detection indicate that these compounds are capable of inducing caspase-dependent apoptosis in a dose-dependent manner. Moreover, these compounds do not have obvious cytotoxicity on normal cells, i.e. $IC_{50} > 50$ μM on HEK-293T cells. Although the molecular targets of this class of compound are yet to be revealed, our current results suggest that this class of compound represents a new possibility for developing drug candidates against acute leukemia.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2020.127114.

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