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Assessment of thiosemicarbazone-containing compounds as potential anti-leukemia agents against P-gp overexpressing drug resistant K562/A02 cells

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P- glycoprotein (P-gp) overexpression is considered to be the leading cause of multidrug resistance (MDR) and failure of chemotherapy for leukemia. In this study, seventeen thiosemicarbazone-containing compounds were prepared and evaluated as potential anti-leukemia agents against drug resistant K562/A02 cell overexpressing P-gp. Among them, (*E*)-*N*-hydroxy-6-(2-(3-nitrobenzylidene)hydrazine-1-carbothioamido)hexanamide could significantly inhibit K562/A02 cells proliferation with an IC_{50} value of 0.96 μM. Interestingly, (*E*)-*N*-hydroxy-6-(2-(3-nitrobenzylidene)hydrazine-1-carbothioamido)hexanamide could dose-dependently increase ROS levels of drug resistant K562/A02 cells, thus displaying a potential collateral sensitivity (CS)-inducing effect and selectively killing K562/A02 cells. Furthermore, (*E*)-*N*-hydroxy-6-(2-(3-nitrobenzylidene)hydrazine-1-carbothioamido)hexanamide possessed potent inhibitory effect on HDAC1 and HDAC6, and could promote K562/A02 cells apoptosis via dose-dependently increasing Bax expression, reducing Bcl-2 protein level, and inducing the cleavage of PARP and caspase3. These present findings suggest that (*E*)-*N*-hydroxy-6-(2-(3-nitrobenzylidene)hydrazine-1-carbothioamido)hexanamide might be a promising lead to discover novel anti-leukemia agents against P-gp overexpressing leukemic cells.

Keywords: Multidrug resistance; P-gp; Thiosemicarbazones; Collateral sensitivity.

Introduction

Multidrug resistance (MDR) is commonly considered as one of the major obstacle to successful chemotherapy for leukemia.[1, 2] Molecularly, the occurrence of MDR is closely related to the ATP-binding cassette (ABC) transporters overexpression, which could efflux various kinds of anti-cancer drugs out of cancer cells, thus resulting in the drug insensitivity. Among the ABC transporter members, P-glycoprotein (P-gp) encoded by MDR1 gene, is the mostly studied and significant one.[3, 4] Therefore, discovery of agents that could overcome P-gp-mediated drug resistance is of great interest.[5]

Nowadays, three generations of inhibitors/modulators have been designed to overcome P-gp induced drug resistance, however, until now, there is no single inhibitor available in clinic mainly because of the high inherent toxicity, poor target specificity, pharmacokinetic interaction, and/or insignificant efficacy.[6, 7] Accordingly, alternative strategies are urgently needed to conquer P-gp-medicated MDR. Notably, resistant cancer cells overexpressing P-gp may be hypersensitive to drugs with particular structure, a phenomenon termed collateral sensitivity (CS).[8] As more compounds were confirmed to have a preferential toxicity towards resistant cancer cells, CS conveyed by P-gp represents a novel and promising strategy to overcome P-gp-mediated MDR.[9, 10]

Recently, plenty of studies showed that compounds with a thiosemicarbazone moiety, such as **Bp44mT** and **Dp44mT** (Figure 1), exhibited preferential toxicities against resistant cancer cells compared to the nonresistant counterparts.[10-12] More importantly, thiosemicarbazones possessed diverse biological activities (ie. anticancer, antivirus) and several agents with a thiosemicarbazone moiety have been evaluated in clinical trials against malignancies including leukemia.[13] Keeping these in mind and considering that histone deacetylases (HDACs) play a significant role in leukemogenesis, and HDACs inhibitors were reported as promising agents for the treatment of leukemia,[14-16] we speculated that hybridizing the thiosemicarbazone moiety with the HDACs inhibitor pharmacophore might result in compounds with more effective anti-leukemia activity against resistant leukemic cells overexpressing P-gp. Thus, seventeen thiosemicarbazone-containing compounds (**3a-q**, Figure 1) with a similar pharmacophore of HDACs inhibior vorinostat (SAHA) were synthesized, and their anti-proliferation activities against P-gp overexpressing K562/A02 cells, and the preliminary mechanisms were subsequently evaluated in this paper.[1]

[1] [1]These authors contributed equally to this work.

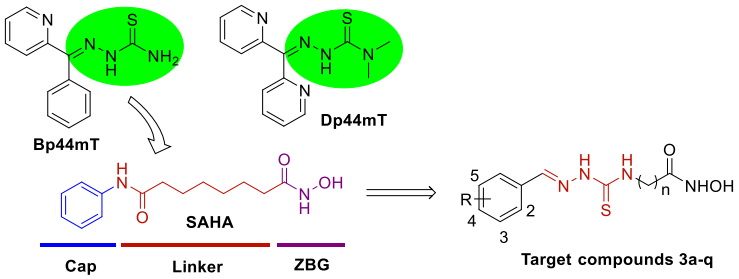
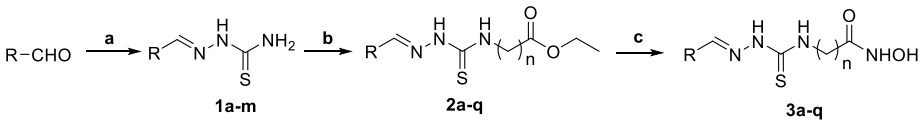


Figure 1. Structures of **Bp44mT**, **Dp44mT** and **3a-q**

Results and Discussion

Chemistry

Synthetic procedure of compounds **3a-q** was depicted in Scheme 1. Briefly, benzaldehyde or benzaldehyde derivatives were condensed with thiosemicarbazide to obtain intermediates **1a-m,** which were subsequently reacted with ethyl 6-bromohexanoate or ethyl 4-bromobutyrate to give intermediates **2a-q,** respectively. The crude products were finally aminolized with hydroxylamine hydrochloride to provide the target compounds **3a-q,** respectively.



3a: R= 2-Cl-C_6H_4, n = 5
3b: R= 2-Br-C_6H_4, n = 5
3c: R= 3-CH_3-C_6H_4, n = 5
3d: R= 3-CF_3-C_6H_4, n = 5
3e: R= 3-F-C_6H_4, n = 5
3f: R= 3-Cl-C_6H_4, n = 5
3g: R= 3-Br-C_6H_4, n = 5
3h: R= 3-NO_2-C_6H_4, n = 5
3i: R= 4-NO_2-C_6H_4, n = 5
3j: R= 4-Br-C_6H_4, n = 5
3k: R= 3,4-$(CH_3O)_2$-C_6H_3, n = 5
3l: R= 3,4,5-$(CH_3O)_3$-C_6H_2, n = 5
3m: R= 3-F-C_6H_4, n = 3
3n: R= 3-Cl-C_6H_4, n = 3
3o: R= 4-NO_2-C_6H_4, n = 3
3p: R= 3,4,5-$(CH_3O)_3$-C_6H_2, n = 3
3q: R= 4-CH_3-C_6H_4, n = 3

Scheme 1. Synthesis of compounds **3a-q**. Reagent and condition: a) Thiosemicarbazide, MeOH, 100 °C, 8 h; b) NaH, 1,4-Dioxane, Ethyl 6-bromohexanoate/Ethyl 4-bromobutyrate, reflux, 10 h; c) EtOH, $NH_2OH{\cdot}HCl$, KOH, -10 °C, 4 h.

Biological evaluation

Inhibitory effect on K562/A02 cells proliferation

To investigate whether the target compounds could overcome P-gp-mediated drug resistance, we initially determined their anti-proliferation effect against P-gp-overexpressed leukemic K562/A02 cell by MTT assay (Table 2). HDACs inhibitor SAHA, anti-cancer drug Adriamycin (ADR) and **Dp44mT** were used as the positive controls and lead control, respectively. Moreover, we also examined their inhibitory effect on the proliferation of parental sensitive leukemic K562 cells to investigate the ability of the target compounds to induce collateral sensitivity (CS) against P-gp. According to literature methods,[17, 18] CS can be quantitatively assessed by calculating the relative resistance (RR) index, which means the ration of IC_{50} values of a compound against resistant and corresponding parental sensitive cells.

Table 1. Effect of compounds **3a-q** on K562/A02 and K562 cell proliferation

	IC50 (μM) [a]				IC50 (μM) [a]		
Compound	K562	K562/A02	RR[b]	Compound	K562	K562/A02	RR[b]
3a	3.87 ± 0.37	4.05 ± 0.41	1.05	**3k**	0.92 ± 0.08	4.55 ± 0.47	4.95
3b	4.81 ± 0.44	4.57 ± 0.51	0.95	**3l**	2.15 ± 0.18	9.79 ± 0.95	4.55
3c	5.72 ± 0.61	5.42 ± 0.43	0.95	**3m**	> 10	> 10	- [c]
3d	4.34 ± 0.36	7.66 ± 0.75	1.77	**3n**	> 10	> 10	-
3e	4.90 ± 0.52	4.63 ± 0.43	0.95	**3o**	7.12 ± 0.83	6.09 ± 0.56	0.86
3f	2.42 ± 0.26	1.12 ± 0.11	0.46	**3p**	> 10	> 10	-
3g	4.23 ± 0.39	4.16 ± 0.27	0.98	**3q**	> 10	> 10	-
3h	2.13 ± 0.23	0.96± 0.089	0.45	SAHA	1.35 ± 0.12	3.13 ± 0.35	2.32
3i	2.63± 0.20	2.51 ± 0.18	0.95	**Dp44mT**	3.43 ± 0.40	3.02 ± 0.29	0.88
3j	6.35 ± 0.59	6.72 ± 0.62	1.06	ADR	0.83 ± 0.095	23.42 ± 4.73	28.21

[a] Triplicate experiments were performed. Data are means ± SD. [b] Relative resistance (RR) = IC50 (resistant cells)/IC50(parental cells). RR < 1 means compounds showed preferential toxicity against MDR cell than parental sensitive cell. RR > 1 indicates cancer cell is resistant. When a compound displaying a RR ≤ 0.5, it is generally defined as a CS-promoting agent. [c] Not determined.

As shown in Table 1, the RR values of ADR and SAHA was 28.21 and 2.32, respectively, indicating that K562/A02 cell was resistant to ADR and SAHA. While, the RR values of the most active compounds were below 1, suggesting that these target compounds displayed selective anti-proliferation effect on P-gp overexpressing K562/A02 cells. In addition, **Dp44mT** displayed a RR value of 0.88 under our experimental conditions, indicating that **Dp44mT** does not display obvious preferential toxicities against K562/A02 cells. Notably, compounds **3f** and **3h** display more potent anti-proliferation activity against K562/A02 cells (IC_{50} values: 1.12 and 0.96 μM, respectively) than positive controls SAHA (IC_{50} value: 3.13 μM), ADR (IC_{50} value: 23.42 μM) and lead control **Dp44mT** (IC_{50} value: 3.02 μM). Interestingly, the RR values of compounds **3f** and **3h** was 0.46 and 0.45, respectively, indicating that both of them might exhibit a potential ability to induce CS against drug resistant K562/A02 cells.

*Effect of compound **3h** on ROS levels in resistant K562/A02 cells*

As mentioned above, one of the leading cause of MDR is the overexpression of P-gp, and CS is regarded as a novel and promising strategy to overcome P-gp-mediated MDR. To clarify the potential mechanism of **3h**, we firstly determined whether compound 3h could affect the expression and/or function of P-gp, and found that **3h** could not affect the efflux function as well as the expression of P-gp (data not shown). Considering that one of the mechanisms underlying CS is the elevated production of ROS in drug resistant cell that overexpresses ABC transporters,[19, 20] we subsequently measured the effect of **3h** on the ROS level in drug resistant K562/A02 cells as well as the parental sensitive K562 cells using DCFHA-DA assay.[21]

As shown in Figure 2, compound **3h** could obviously increase the intracellular ROS in K562/A02 cells (Fig. 2A, blue bar) at a dose depended manner. And the ROS levels were relatively higher than that in parental sensitive K562 cells (Fig. 2A, red bar). As expected, ROS scavenger Nacetyl-cysteine (NAC) could remarkably diminish the levels of ROS in compound **3h**-treated K562/A02 cells. In addition, data showed that the well-known P-gp inhibitor tariquidar (Tar) could significantly reduce the levels of ROS in P-gp overexpressing K562/A02 cells incubated with **3h**, but have little effect on sensitive K562 cells, indicating that the increasing effect of **3h** on the levels of ROS in K562/A02 cells was P-gp-dependent. Notably, non-toxic NAC could dramatically attenuate the anti-proliferation effect compound **3h** on K562/A02 cells (Fig. 2B), indicating that the cytotoxicity and CS-promoting effect of compound **3h** on P-gp overexpressing K562/A02 cells might rely on the increased production of the intracellular ROS, at least partly.

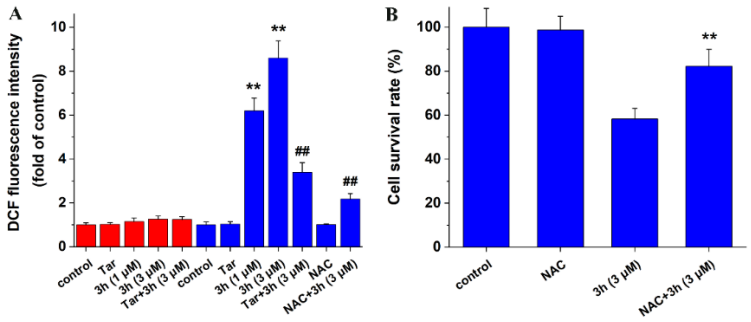


Figure 2. Effects of **3h** on K562/A02 (blue bar) and K562 (red bar) ROS levels and viability. (A) Reactive oxygen species (ROS) level in K562/A02 cells (blue bar) and K562 cells (red bar) were measured using the DCFHA-DA assay. $^{**}P < 0.01$ *vs*. control group (blue bar). $^{\#\#}P < 0.01$ *vs*. the **3h** (3.0 μM) group in K562/A02 cells (blue bar). (B) K562/A02 cells were incubated in the absence or presence of **3h** (3.0 μM) for 24 h, the cell viability was then determined by MTT assay. In the case of inhibition, NAC (10 mM) or Tar (5.0 μM) and **3h** (3.0 μM) were incubated with K562/A02 cells together. $^{**}P < 0.01$ *vs*. the **3h** (3.0 μM) group.

*Effect of compound **3h** on K562/A02 cells apoptosis*

Subsequently, Annexin V/PI assay was employed to investigate whether compound **3h** could lead to enhanced cell apoptosis, a major mechanism of anti-proliferation.[22, 23] Data showed that (Fig. 3), after the 48 h treatment of K562/A02 cells with **3h** (1.0 or 3.0 μM), the percentage of early and late apoptotic cells were significantly increased to 64.94% and 72.84%, respectively, indicating that compound **3h** possessed an apoptosis-inducing effect on K562/A02 cells.

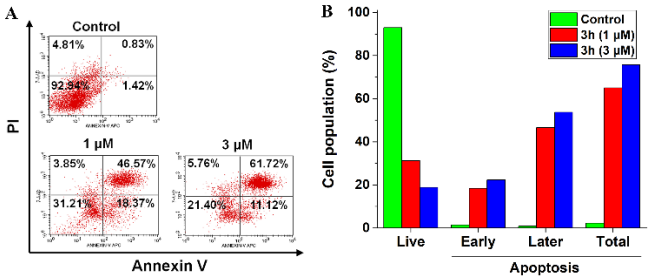


Figure 3. Compound **3h** induced the K562/A02 cells apoptosis (A) After 48 h treatment of K562/A02 cells with or without **3h** (1.0 or 3.0 μM), cells were collected and stained using Annexin V/PI. Early and late (Annexin-V+/PI−) apoptotic K562/A02 cells were analyzed by flow cytometry; (B) Percentage of cell distribution.

*Effect of **3h** on HDACs and the expression of apoptosis-related proteins in K562/A02 cells*

HDACs play significant roles during the leukemogenesis.[14-16] In order to investigate the effect of the active compound **3h** on HDACs activity, the fluorescent-based HDAC biochemical activity were subsequently determined accordingly our previous method.[24] Thiosemicarbazone-containing compound **Dp44mT** and SAHA were used as lead and positive controls, respectively.

As summarized in Table 2, **Dp44mT** showed little inhibitory activities on HDACs. While compound **3h** exhibited potent inhibitory effect on HDAC1 and HDAC6 activities with IC_{50} values even lower than those of classical HDACs inhibitor SAHA (28.2 nM and 24.3 nM *vs.* 37.7 nM and 33.1 nM, respectively). However, **3h** could not inhibit the activity of HDAC8, indicating that **3h** might exhibit a selective inhibitory effect on HDACs activities.

Table 2. Inhibitory effect of target compound **3h** on HDAC1, 6 and 8 activity.

	IC50 (nM) [a]		
Compounds	**HDAC1**	**HDAC6**	**HDAC8**
3h	28.2 ± 2.1	24.3 ± 3.2	> 5000
SAHA	37.7 ± 3.7	33.1 ± 2.9	> 5000
Dp44mT	> 5000	> 5000	> 5000

[a] Three independent experiments were performed. Values are mean ± SD.

To further confirm the HDACs inhibitory effect and apoptosis induction effect of the active compound **3h** on drug resistant K562/A02 cells, we determined the expression of Ac-H_3, Ac-H_4 (well-known substrates of HDAC1, 2 and 3),[25] and several key apoptotic proteins, including Bcl-2, Bax, and cleavage state of caspase3 and PARP (apoptosis markers) in K562/A02 cells treated with **3h** by western blot detection.

As depicted in Figure 4, **3h** could dose-dependent increased Ac-H_3, Ac-H_4, and pro-apoptotic Bax proteins expression while reducing the expression of anti-apoptotic Bcl-2. In addition, compared to control group, **3h** could more obviously induce the cleavage of caspase3 and PARP. Together, these data confirmed that **3h** exhibited potential HDACs inhibitory activity and apoptosis-inducing effect against K562/A02 cells.

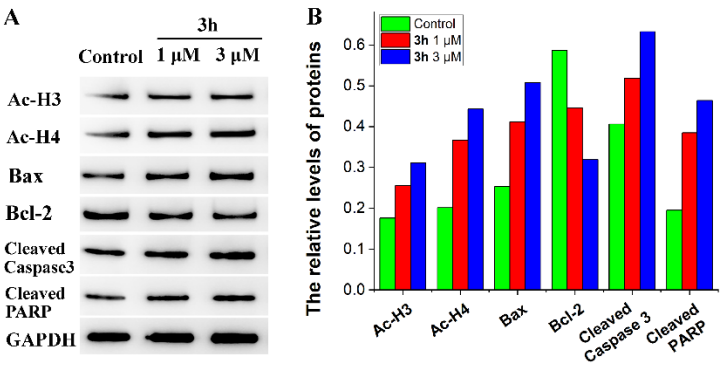


Figure 4. **3h** affected the expression of Ac-H_3, Ac-H_4 and apoptosis-related proteins. (A) Apoptosis-related proteins and Ac-H_3, Ac-H_4 were determined by western blotting assay. After 48 h treatment of K562/A02 cells with or without **3h** (1.0 or 3.0 μM), cells were harvested and lysed to detect related proteins expression. (B) Density ratios of Ac-H_3, Ac-H_4, apoptosis-related proteins to GADPH.

Structure-Activity Relationship

Based on the data in Table 1, the structure-activity relationships (SAR) of the target compounds on K562/A02 cells proliferation were preliminarily drawn as follows: the length of the carbon chain linked to the thiosemicarbazone and hydroxamic acid moiety could significantly affect the anti-proliferation

activity. Compound with a five-carbon chain showed more potent anti-proliferation activity than compound with a three-carbon chain (**3e** *vs.* **3m**; **3f** *vs.* **3n**; **3i** *vs.* **3o**; **3l** *vs.* **3p**). In addition, compound with a meta substituent on the benzene ring exhibited more potent anti-proliferation activity than compound with an ortho or para substitution (**3f** *vs.* **3a**; **3g** *vs.* **3b**, **3j**; **3h** *vs.* **3i**). Notably, the type of meta substituent could also obviously affect the anti-proliferation activity. Among them, nitro substituent was the best, followed by chlorine, and the trifluoromethyl and methyl groups were the worst (**3h**, **3f** *vs.* **3c**, **3d**).

Conclusions

In summary, seventeen thiosemicarbazone-containing compounds were prepared, and subsequently their anti-leukemia effect on K562/A02 cells were determined. Among them, **3h** possessed significant anti-proliferation activity against K562/A02 cells with an IC_{50} value of 0.96 μM. Interestingly, compound **3h** exhibited a potential CS-inducing effect by increasing the level of intracellular ROS in K562/A02 cells, thus displaying a preferential toxicity against K562/A02 cells. In addition, **3h** could potently inhibit HDAC1 and HDAC6, and induce K562/A02 cells apoptosis by dose-dependently affecting the expression of apoptosis-related proteins Bax and Bcl-2, and inducing the cleavage of caspase3 and PARP. Considering the potent anti-proliferation activity, CS and apoptosis-inducing effects, and inhibitory effects on HDAC1 and HDAC6, compound **3h** might be a promising lead to discover new anti-cancer agents towards leukemic cells overexpressing P-gp.

Experimental Section

Chemical analysis

Column chromatography (thin layer chromatography or 200-300 mesh silica gel 60) were used to purify synthesized compounds. Melting point of each compound was determined using an apparatus of model YRT-3 (uncorrected). Chemical structure of each target compound was confirmed by NMR (400 MHz for ^{1}H and 100 MHz for ^{13}C, JEOL), MS (Agilent 6530 QTOF and Agilent 6460 spectrometer), and IR (Shimadzu, FTIR-8400S) routinely. Chemical structure analysis was performed in Xuzhou Medical University of Public Experimental Research Center. All the target compounds **3a-q** were new compounds. High-performance liquid chromatography (Agilent 1260 HPLC instrument, equipped with 150*4.6 mm Daicel CHIRALCEL OD-RH column and UV detection at 254 nm) was used to measure the purity of each target compound. The mobile phase consistent of a binary gradient of MeOH (A) and H2O with 0.1% formic acid (B). The flow rate was 0.8 mL/min.

*Synthesis of target compounds **3a-q***

Benzaldehyde (500 mg) or various substituted benzaldehyde and thiosemicarbazide (1.0 eq) was dissolved in methanol (100 mL). After 8 h stirring under nitrogen (N_2, gas) at 100 °C, the solution was filtered to obtain crude products **1a-m**, respectively, which were then dissolved in 50 mL Dioxane. After 5 min stirring at 0 °C, 1 eq NaH was added portionwise (keep the solution at 0 °C).Then 2 eq ethyl 6-bromohexanoate or ethyl 4-bromobutyrate was added to the reaction solution. After 10 h stirring at 120 °C under nitrogen, the mixture was cooled down to room temperature. Then water was added to the mixture, and dispersed with ethyl acetate. Organic phase was dried with anhydrous Na_2SO_4, and removed by evaporation to afford the corresponding intermediates **2a-q**, respectively. The crude products **2a-q** was dissolved in 50 mL ethanol. After cooling to -10 °C, hydroxylamine hydrochloride (20 eq) and potassium hydroxide (21 eq) were added to the cooled solution, and stirred for another 4 h. Finally, 10 % HCl was added to the solution (neutral pH), which was extracted with ethyl acetate, and dried over anhydrous Na_2SO_4. After removal of the solvent, crude products were finally purified using column chromatography to give the target compounds **3a-q**, respectively. CH_2Cl_2-MeOH (50:1-30:1, v/v) was selected as the eluent. The spectroscopic data of compounds **3a-q** were shown in the supporting information.

Cytotoxicity assay

K562 and K562/A02 cells (KeyGen Co. Ltd., Nanjing, China) were cultured as described previously [26, 27]. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the anti-proliferation activity of compounds **3a-q** [28]. Briefly, 1×10^4 K562 or K562/A02 cells were seeded in 96-well culture plates and cultured for 24 h to maintain the cells in exponential growth phase. Then, cells were incubated for another 72 h with or without the test compounds at indicated concentrations, respectively. Then, MTT (final concentration of each well: 5 mg/mL) was added into each well at 4 h before the test. The insoluble formazan was collected and dissolved in dimethylsulfoxide (DMSO, 0.5%), and absorbance was detected at 490 nm using a microplate enzyme-linked immunosorbent assay reader. The inhibition rate of cell growth (%) was calculated as [A490 (mean negative control group)-A490 (mean compound treatment group)/(A490 (mean negative control group)-A490 (mean blank control group)] × 100%. In case of inhibition, cells were incubated with Nacetyl-cysteine (NAC, 10 mM) and the test compounds.

Determination of ROS levels in K562 or K562/A02 cells

After 12 h incubation of K562 or K562/A02 cells with **3h** (1.0 or 3.0 μM) or control (DMSO), cells (1×10^6 cells/mL) were collected, and DCFHA-DA (3 mM) was used to stain the cells in the dark at 37 °C for 20 min. After wash by PBS, FITC fluorescent intensity was subsequently determined via FACS Calibur flowcytometer (Becton-Dickinson). ROS level in cells treated with **3h** was normalized to the relative control. In case of inhibition, cells were incubated with Nacetyl-cysteine (NAC, 10 mM) or Tar (5.0 μM) and the test compound **3h** (3.0 μM).

Effect on HDAC1, 6 and 8 activity

HDAC1, 6 and 8 activity were determined using the HDAC fluorimetric activity assay kit (Signalchem Inc.) according to manufacturer's instruction. Briefly, HDAC1, 6 or 8 enzyme solution was incubated with compound **3h** at indicated concentrations in the presence of 20 μM Boc-Lys (Ac)-AMC (HDAC substrate) at 37 °C for 30 min. Then the reaction was stopped by adding the lysine developer. Finally, the fluorescence intensities were recorded in a SpectraMax M5 microtiter plate reader (emission: 460 nm; excitation: 360 nm).

Analysis of Cells Apoptosis

An apoptosis assay kit was used to perform Annexin V staining (KeyGen, Nanjing, China). Briefly, after 48 h incubation of K562/A02 cells with or without **3h** (1.0 or 3.0 μM), cells were washed with PBS, and reincubated with annexin V binding buffer (500 μL). Subsequently, after the harvesting and washing, cells were stained in the darkness for 15 min with5 μL propidium iodide and annexin V-FITC. Finally, a FACS Calibur flow cytometer was served to determine the percentage of apoptotic cells (Becton-Dickinson, San Jose, USA).

*Effect of **3h** on Ac-H3, Ac-H4 and apoptotic proteins expression*

After separation of K562/A02 cells lysate with SDS-PAGE (40 μg protein/lane), each protein was transferred to the PVDF membrane via electroblotting. Subsequently, each blot was blocked with milk (5%) and probed with antibody against acetyl-histone H3/4, Bcl-2, Bcl-xl, cleaved caspase-3 and PARP (Sigma-Aldrich), respectively. After washing with TBST, each blot was incubated for 2 h with secondary antibodies. Finally, each blot was detected and imaged with ECL and G:BOX chemiXR5 digital imaging system, respectively.

Supplementary Material

Supporting information for this article is available on line.

Acknowledgements

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Author Contribution Statement

Xiaoke Gu and Mingyu Guan conducted the synthesis experiments, characterized the compounds, and prepared the first draft. Chunyu Jiang, Qinghua Song, Xin Li, Nan Sun and Jing Chen performed biological tests and analyzed the data. Xiaoke Gu and Jingying Qiu designed and supervised the project and wrote the article. The final revision of the manuscript was revised by all the authors.

Reference

[1] M. Kairuki, Q. Q. Qiu, M. B. Pan, Q. F. Li, J. Q. Zhou, H. Ghaleb, W. L. Huang, H. Qian, C. Jiang, 'Designed P-glycoprotein inhibitors with triazol-tetrahydroisoquinoline-core increase doxorubicin-induced mortality in multidrug resistant K562/A02 cells', *Bioorg. Med. Chem.* **2019**, *27*, 3347-3357.

[2] S. M. Ibrahim, S. Bakhashab, A. M. Ilyas, P. N. Pushparaj, S. Karim, J. A. Khan, A. M. Abuzenadah, A. G. Chaudhary, M. H. Al-Qahtani, F. Ahmed, 'WYE-354 restores Adriamycin sensitivity in multidrug-resistant acute myeloid leukemia cell lines', *Oncol. Rep.* **2019**, *41*, 3179-3188.

[3] S. Dei, M. N. Romanelli, D. Manetti, N. Chiaramonte, M. Coronnello, M. Salerno, E. Teodori, 'Design and synthesis of aminoester heterodimers containing flavone or chromone moieties as modulators of P-glycoprotein-based multidrug resistance (MDR)', *Bioorg. Med. Chem.* **2018**, *26*, 50-64.

[4] R. W. Robey, K. M. Pluchino, M. D. Hall, A. T. Fojo, S. E. Bates, M. M. Gottesman, 'Revisiting the role of ABC transporters in multidrug-resistant cancer', *Nat. Rev. Cancer* **2018**, *18*, 452-464.

[5] V. Lopes-Rodrigues, A. Oliveira, M. Correia-da-Silva, M. Pinto, R. T. Lima, E. Sousa, M. H. Vasconcelos, 'A novel curcumin derivative which inhibits P-glycoprotein, arrests cell cycle and induces apoptosis in multidrug resistance cells', *Bioorg. Med. Chem.* **2017**, *25*, 581-596.

[6] Y. Ma, D. Yin, J. Ye, X. Wei, Y. Pei, X. Li, G. Si, X. Y. Chen, Z. S. Chen, Y. Dong, F. Zou, W. Shi, Q. Qiu, H. Qian, G. Liu, 'Discovery of Potent Inhibitors against P-Glycoprotein-Mediated Multidrug Resistance Aided by Late-Stage Functionalization of a 2-(4-(Pyridin-2-yl)phenoxy)pyridine Analogue', *J. Med. Chem.* **2020**, *63*, 5458-5476.

[7] B. Wang, L. Y. Ma, J. Q. Wang, Z. N. Lei, P. Gupta, Y. D. Zhao, Z. H. Li, Y. Liu, X. H. Zhang, Y. N. Li, B. Zhao, Z. S. Chen, H. M. Liu, 'Discovery of 5-Cyano-6-phenylpyrimidin Derivatives Containing an Acylurea Moiety as Orally Bioavailable Reversal Agents against P-Glycoprotein-Mediated Mutidrug Resistance', *J. Med. Chem.* **2018**, *61*, 5988-6001.

[8] T. Efferth, M. E. M. Saeed, O. Kadioglu, E. J. Seo, S. Shirooie, A. T. Mbaveng, S. M. Nabavi, V. Kuete, 'Collateral sensitivity of natural products in drug-resistant cancer cells', *Biotechnol. Adv.* **2020**, *38*, 107342.

[9] A. Furedi, S. Toth, K. Szebenyi, V. F. Pape, D. Turk, N. Kucsma, L. Cervenak, J. Tovari, G. Szakacs, 'Identification and Validation of Compounds Selectively Killing Resistant Cancer: Delineating Cell Line-Specific Effects from P-Glycoprotein-Induced Toxicity', *Mol. Cancer Ther.* **2017**, *16*, 45-56.

[10] V. F. S. Pape, S. Toth, A. Furedi, K. Szebenyi, A. Lovrics, P. Szabo, M. Wiese, G. Szakacs, 'Design, synthesis and biological evaluation of thiosemicarbazones, hydrazinobenzothiazoles and arylhydrazones as anticancer agents with a potential to overcome multidrug resistance', *Eur. J. Med. Chem.* **2016**, *117*, 335-354.

[11] A. E. Stacy, D. Palanimuthu, P. V. Bernhardt, D. S. Kalinowski, P. J. Jansson, D. R. Richardson, 'Structure-Activity Relationships of Di-2-pyridylketone, 2-Benzoylpyridine, and 2-Acetylpyridine Thiosemicarbazones for Overcoming Pgp-Mediated Drug Resistance', *J. Med. Chem.* **2016**, *59*, 8601-8620.

[12] G. Szakacs, M. D. Hall, M. M. Gottesman, A. Boumendjel, R. Kachadourian, B. J. Day, H. Baubichon-Cortay, A. Di Pietro, 'Targeting the Achilles heel of multidrug-resistant cancer by exploiting the fitness cost of resistance', *Chem. Rev.* **2014**, *114*, 5753-5774.

[13] M. D. Hall, K. R. Brimacombe, M. S. Varonka, K. M. Pluchino, J. K. Monda, J. Li, M. J. Walsh, M. B. Boxer, T. H. Warren, H. M. Fales, M. M. Gottesman, 'Synthesis and structure-activity evaluation of isatin-beta-thiosemicarbazones with improved selective activity toward multidrug-resistant cells expressing P-glycoprotein', *J. Med. Chem.* **2011**, *54*, 5878-5889.

[14] L. Y. Yang, Q. Qiu, M. H. Tang, F. Wang, Y. Y. Yi, D. N. Yi, Z. Yang, Z. J. Zhu, S. J. Zheng, J. H. Yang, H. Y. Pei, L. Zheng, Y. Chen, L. P. Gou, L. Y. Luo, X. Deng, H. Y. Ye, Y. G. Hu, T. Niu, L. J. Chen, 'Purinostat Mesylate Is a Uniquely Potent and Selective Inhibitor of HDACs for the Treatment of BCR-ABL-Induced B-Cell Acute Lymphoblastic Leukemia', *Clin. Cancer Res.* **2019**, *25*, 7527-7539.

[15] M. Mehrpouri, A. Safaroghli-Azar, A. Pourbagheri-Sigaroodi, M. Momeny, D. Bashash, 'Anti-leukemic effects of histone edeacetylase (HDAC) inhibition in acute lymphoblastic leukemia (ALL) cells: Shedding light on mitigating effects of NF-kappa B and autophagy on panobinostat cytotoxicity', *Eur. J. Pharmacol.* **2020**, *875*.

[16] M. Lernoux, M. Schnekenburger, H. Losson, K. Vermeulen, H. Hahn, D. Gerard, J. Y. Lee, A. Mazumder, M. Ahamed, C. Christov, D. W. Kim, M. Dicato, G. Bormans, B. W. Han, M. Diederich, 'Novel HDAC inhibitor MAKV-8 and imatinib synergistically kill chronic myeloid leukemia cells via inhibition of BCR-ABL/MYC-signaling: effect on imatinib resistance and stem cells', *Clin. Epigenetics* **2020**, *12*, 1-26.

[17] C. Ramalhete, S. Mulhovo, H. Lage, M.-J. U. Ferreira, 'Triterpenoids from Momordica balsamina with a collateral sensitivity effect for tackling multidrug resistance in cancer cells', *Planta Med.* **2018**, *84*, 1372-1379.

[18] J. A. Mendes, P. Merino, T. Soler, E. J. Salustiano, P. R. Costa, M. Yus, F. Foubelo, C. D. Buarque, 'Enantioselective Synthesis, DFT Calculations, and Preliminary Antineoplastic Activity of Dibenzo 1-Azaspiro [4.5] decanes on Drug-Resistant Leukemias', *J. Org. Chem.* **2019**, *84*, 2219-2233.

[19] C. Riganti, R. Giampietro, J. Kopecka, C. Costamagna, F. S. Abatematteo, M. Contino, C. Abate, 'MRP1-Collateral Sensitizers as a Novel Therapeutic Approach in Resistant Cancer Therapy: An In Vitro and In Vivo Study in Lung Resistant Tumor', *Int. J. Mol. Sci.* **2020**, *21*, 3333.

[20] Q. Cui, J. Q. Wang, Y. G. Assaraf, L. Ren, P. Gupta, L. Wei, C. R. Ashby, Jr., D. H. Yang, Z. S. Chen, 'Modulating ROS to overcome multidrug resistance in cancer', *Drug Resist. Update.* **2018**, *41*, 1-25.

[21] X. Gu, J. Chen, Y. Zhang, M. Guan, X. Li, Q. Zhou, Q. Song, J. Qiu, 'Synthesis and assessment of phenylacrylamide derivatives as potential anti-oxidant and anti-inflammatory agents', *Eur. J. Med. Chem.* **2019**, *180*, 62-71.

[22] Y. Ling, W.-J. Gao, C. Ling, J. Liu, C. Meng, J. Qian, S. Liu, H. Gan, H. Wu, J. Tao, 'β-Carboline and N-hydroxycinnamamide hybrids as anticancer agents for drug-resistant hepatocellular carcinoma', *Eur. J. Med. Chem.* **2019**, *168*, 515-526.

[23] Y. Hu, K. Yu, G. Wang, D. Zhang, C. Shi, Y. Ding, D. Hong, D. Zhang, H. He, L. Sun, 'Lanatoside C inhibits cell proliferation and induces apoptosis through attenuating Wnt/β-catenin/c-Myc signaling pathway in human gastric cancer cell', *Biochem. Pharmacol.* **2018**, *150*, 280-292.

[24] X. Gu, X. Li, M. Guan, C. Jiang, Q. Song, N. Sun, Y. Zou, Q. Zhou, J. Chen, J. Qiu, 'Discovery of thiosemicarbazone-containing compounds with potent anti-proliferation activity against drug-resistant K562/A02 cells', *Bioorg. Med. Chem. Lett* **2020**, *30*, 127638-127642.

[25] X. Li, E. S. Inks, X. Li, J. Hou, C. J. Chou, J. Zhang, Y. Jiang, Y. Zhang, W. Xu, 'Discovery of the First N-Hydroxycinnamamide-Based Histone Deacetylase 1/3 Dual Inhibitors with Potent Oral Antitumor Activity', *J. Med. Chem.* **2014**, *57*, 3324-3341.

[26] Z. G. Ren, X. K. Gu, B. Lu, Y. Q. Chen, G. J. Chen, J. N. Feng, J. Z. Lin, Y. H. Zhang, H. Peng, 'Anticancer efficacy of a nitric oxide-modified derivative of bifendate against multidrug-resistant cancer cells', *J. Cell. Mol. Med.* **2016**, *20*, 1095-1105.

[27] X. K. Gu, Z. J. Huang, Z. G. Ren, X. B. Tang, R. F. Xue, X. J. Luo, S. X. Peng, H. Peng, B. Lu, J. D. Tian, Y. H. Zhang, 'Potent Inhibition of Nitric Oxide-Releasing Bifendate Derivatives against Drug-Resistant K562/A02 Cells in Vitro and in Vivo', *J. Med. Chem.* **2017**, *60*, 928-940.

[28] S. Ji, S. Tang, K. Li, Z. Li, W. Liang, X. Qiao, Q. Wang, S. Yu, M. Ye, 'Licoricidin inhibits the growth of SW480 human colorectal adenocarcinoma cells in vitro and in vivo by inducing cycle arrest, apoptosis and autophagy', *Toxicol. Appl. Pharm.* **2017**, *326*, 25-33.

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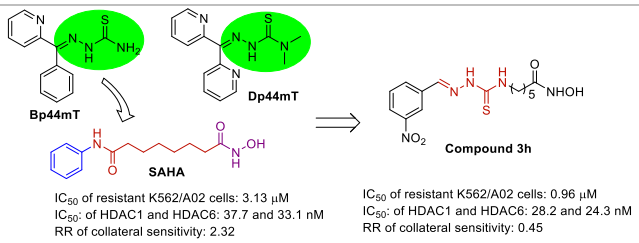

Twitter Text

Compound **3h** showed potent anti-leukemia effect on P-gp overexpressing resistant K562/A02 cells by increasing ROS level, regulating apoptosis-related proteins expression and inhibiting HDAC1/6 activity.