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#### Article

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# A novel triapine derivative induces copperdependent cell death in hematopoietic cancers

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KEYWORDS: hematopoietic cancers, triapine, copper, mitochondrial ROS, TXNIP.

ABSTRACT

Triapine, an iron chelator that inhibits ribonucleotide reductase, has been evaluated in clinical trials for cancer treatment. Triapine in combination with other chemotherapeutic agents shows promising efficacy in certain hematologic malignancies; however, it is less effective against many advanced solid tumors, probably due to the unsatisfactory potency and pharmacokinetic properties. In this report, we developed a triapine derivative IC25 (10) with potent anti-tumor activity. 10 preferentially inhibited the proliferation of hematopoietic cancers by inducing mitochondria ROS production and mitochondrial dysfunction. Unlike triapine, 10 executed cytotoxic action in a copper-dependent manner. 10-induced upexpression of TXNIP resulted in decreased TRX activity to permit JNK and p38 activation and ultimately led to execution of cell death program. Remarkedly, 10 showed good bioavailability and inhibited tumor growth in mouse xenograft models. Taken together, our study identifies compound 10 as a copper-dependent antitumor agent, which may be applied to the treatment of hematopoietic cancers.

#### INTRODUCTION

Acute leukemia is one of the most prevalent cancer types in both early childhood and adult population. Depending on the various subtypes of leukemia and the underlying genetic mutations, the long-term survival varies considerably.<sup>1,</sup> <sup>2</sup> Over last decades, the treatment of leukemia has progressed slowly and the mainstay is still chemotherapy. In spite of intensive combination conventional standard therapies and allogeneic hematopoietic stem cell transplantation, hematopoietic cancers remain difficult to be treated.<sup>3-5</sup> Consequently, there is an urgent need for the development of more effective therapies for leukemia, with a view to boosting overall survival and improving treatment outcomes.

3-Aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP, triapine) shows broad-spectrum antitumor activity *in vitro* and is currently being evaluated in multiple clinical trials. Early results revealed that administration of triapine alone could not yield survival benefits for cancer patients,<sup>6-9</sup> and this may be attributed to its weak potency. A combination of triapine with other chemotherapeutic agents, however, has shown promising disease control in myeloproliferative neoplasms, cervical and vaginal cancers.<sup>10-12</sup> Several hypotheses have been advanced

concerning the mechanisms involved in the antitumor activity of triapine. Among them, what is more important is that triapine can promote reactive oxygen species (ROS) production mediated by forming complex with iron.<sup>13-16</sup> ROS, as natural products of cellular metabolism, can damage DNA, proteins and lipids. Accumulating evidence suggests that regulation of ROS level and causing oxidative stress are intelligent strategies for cancer therapy.<sup>17-18</sup>

Although triapine is a well-known chelator of transition metal ions such as iron (Fe), copper (Cu) and zinc (Zn), the exact metal ion partners relevant to the biological activities is yet to be identified.<sup>19-20</sup> Cu complexes have long been predicted as biologically active forms due to its intracellular redox cycling potential. Copper is a redox-active transition metal which plays important roles as cofactors in various enzymes that function in antioxidant defense, mitochondrial respiration, neurotransmitter synthesis and a variety of biochemical processes.<sup>21-22</sup> However, the uncontrolled accumulation of copper can also be harmful to cells by inducing oxidative stress, which damages cellular components and ultimately leads to cell death. Thorough systems of copper regulation and trafficking are required to

satisfy the cellular copper requirement and simultaneously minimize the potential toxicity.<sup>23-25</sup>

Cellular thiols are susceptible to several oxidants, and the thioredoxin (TRX) has a conserved catalytic site containing cysteine that can be oxidized to cysteine disulfide reversibly so as to regulate thiol redox balance. TRX is a scavenger of ROS, protecting cells from oxidation-induced apoptosis.<sup>26-28</sup> The reduced form of TRX directly binds to the N-terminal non-catalytic region of apoptosis signaling kinase-1 (ASK1) and inhibits its kinase activity. The oxidation of reduced TRX disjoins ASK1 and activates c-Jun N-terminal kinase (JNK) and p38 MAPK pathway.<sup>29-33</sup> Thioredoxin-interacting protein (TXNIP) is a pro-oxidative stress, proinflammatory and pro-apoptotic protein that binds to TRX and inhibits its thiolreducing and oxidant-scavenging activity, thereby triggering cellular oxidative stress and apoptosis.<sup>29, 33</sup> Translocation of TXNIP to mitochondria is the other well-known event following ROS inducer, and TXNIP binds to TRX2 leading to mitochondrial dysfunction.34-36

In this study, we designed and synthesized a series of triapine analogs to improve its potency. Among them, compound **10** exhibited potent anti-tumor effect and favorable PK properties. **10**-induced cell death was associated with increased levels of mitochondrial ROS mediated by copper. Moreover, it further induced up-expression of TXNIP and inhibited TRX activity, along with mitochondrial dysfunction, and then activated JNK and p38 MAPK pathway. Importantly, **10** exhibited anti-tumor activity in MV4;11 leukemia xenograft models *in vivo*, suggesting the potential of **10** for the treatment of hematopoietic cancers.

#### RESULTS

**Design novel triapine derivatives with potent anti-tumor activity.** Triapine has been proposed as a metal chelator through N\*-N\*-S\* terdentate coordination system (Figure 1A). Based on the binding mode, we designed three triapine derivatives, **1**, **2** and **3** (Scheme 1), by cyclization of the NH<sub>2</sub> group and aromatization of the thiocarbamide moiety (Figure 1A). We then compared the antitumor activity of the three compounds to that of triapine in two hematopoietic

tumor cell lines KG1a and KMS11 and two solid tumor cell lines A549 and Hela. Compound **1** and **2** inhibited the proliferation of tumor cells more potent than triapine, while **3** showed much diminished inhibition, suggesting the formation of 5-member ring disrupted the antitumor activity (Figure 1B). Moreover, both **1** and **2** were more effective for hematopoietic tumor cells than solid tumor cells (Figure 1B). We further profiled **1** and **2** in additional four leukemia cell lines and revealed that the antiproliferative activity of **1** was more potent than that of **2**, suggesting that benzothiazole is more favorable than benzimidazole (Figure 1C).



Figure 1. The antiproliferative activity of triapine derivatives 1, 2 and 3. (A) Chemical structures of compound 1, 2 and 3. (B) The antiproliferative activities of 1, 2, 3 and triapine were evaluated in two hematopoietic tumor cell lines (KG1a and KMS11) and two solid tumor cell lines (A549 and Hela) with a CellTiter-Glo<sup>™</sup> Luminescent cell viability assay 48 h post-treatment, and the cell viability was measured compared to that of vehicle-treated cells. (C) SEM, HL60, KOPN8 and MV4;11 cells were treated with compound 1 and 2 for 48 h, cell viability was analyzed.

We next investigated the structure-activity relationship (SAR) of **1** (Table 1). Compound **4**, which lacks the N\*-N\*-S\* terdentate coordination system, totally abolished the cytotoxicity, indicating that chelation with metal is indispensable for the antitumor activity of triapine analogs. Replacement of fused cyclohexane with tetrahydropyran resulted in **5**, which is much less potent and less selective for MV4;11 cells over normal human umbilical vein endothelial cells (HUVEC). A methyl substitution on hydrazine (**6**) led to complete loss of antitumor activity.

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|--------------|---|--------|-----------------------|-----------------------|-----------------|--------|
| Compou<br>nd | Х   | Y      | R1                    | MV4;11)               | HUVEC)          |        |
| Triapine     |   |        |                       | 967.6 ± 108.4         | 3,448 ± 538     | 3.6    |
| 1            | Ν   | $CH_2$ | Н                     | 8.9 ± 0.3             | 908.1 ± 29.4    | 102.0  |
| 4            | С   | $CH_2$ | Н                     | > 50,000              | 15,445 ±<br>604 | < 0.31 |
| 5            | Ν   | 0      | Н                     | 197.1 ± 33.7          | 698 ± 28        | 3.5    |
| 6            | Ν   | $CH_2$ | CH <sub>3</sub>       | > 50,000              | 22,272 ±<br>341 | < 0.45 |

<sup>a</sup>: SF (selectivity factor): IC<sub>50</sub> values in HUVEC/ IC<sub>50</sub> values in MV4;11.

Given these results, we mainly focused on the optimization of benzothiazole in the right part (Table 2, Scheme 1). Installation of methoxyl (7) or fluoride (13) to 6-position ( $R_2$ ) of benzothiazole dramatically compromised the cytotoxic activity, suggesting that 6-position substitution was not tolerated. Fluoro or methoxyl substitution on other positions of benzothiazole slightly decreased the potency (8, 9, 14-16). Interestingly, dimethoxyl substitution 10 was more potent ( $IC_{50} = 3.6$ nM) and the selectivity over HUVEC was much better than 1. 1,3-dioxolane analog 11 exhibited similar selectivity as 1, however, the potency was slightly

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decreased. EWG group trifluoromethoxyl (12) significantly compromised the potency to 179.5 nM. Alkyl groups such as methyl (17), dimethyl (18) and indane analog (19) did not benefit the potency either.

Scheme 1. Synthesis of triapine derivatives



Reagents and conditions: (a) KSCN, Br<sub>2</sub>, AcOH, 0  $^{\circ}$ C-rt, 3 h.<sup>37</sup> (b) 80% Hydrazine hydrate, conc. HCl, Ethylene glycol, 150  $^{\circ}$ C, 5 h.<sup>38</sup> (c) NaCS<sub>2</sub>OEt, DMF, 5  $^{\circ}$ C, 4 h; SOCl<sub>2</sub>, CH<sub>2</sub>Cl<sub>2</sub>, 0  $^{\circ}$ C-rt, 2 h.<sup>39</sup> (d) 80% Hydrazine hydrate, EtOH, 70  $^{\circ}$ C, 5 h. (e) 6,7-dihydroquinolin-8(5H)-one, MeOH, reflux, 5 h.





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| 1  | Н        | Н                  | Н                                     | Н | 8.9 ± 0.3     | 908.1 ±<br>29.4  | 102.0 |
|----|----------|--------------------|---------------------------------------|---|---------------|------------------|-------|
| 7  | OCH<br>3 | н                  | н                                     | Н | 1,436 ± 37    | 3,537 ± 444      | 2.5   |
| 8  | Н        | OCH<br>3           | Н                                     | Н | 37.3 ± 3.6    | 427.4 ±<br>121.5 | 11.5  |
| 9  | Н        | Н                  | OCH<br>3                              | Н | 33.4 ± 0.4    | 1,040 ± 92       | 31.1  |
| 10 | Н        | OCH<br>3           | OCH<br>3                              | Н | $3.6 \pm 0.6$ | 924.6 ±<br>80.6  | 256.8 |
| 11 | Н        | -OC                | H <sub>2</sub> O-                     | Н | 22.3 ± 3      | 2,171 ± 117      | 97.4  |
| 12 | Н        | Н                  | $OCF_3$                               | Н | 179.5 ± 49.4  | 609 ± 21         | 3.4   |
| 13 | F        | Н                  | Н                                     | Н | 1,988 ± 43    | 4,977 ± 121      | 2.5   |
| 14 | Н        | F                  | Н                                     | Н | 33.5 ± 8.2    | 2,649 ± 418      | 79.1  |
| 15 | Н        | Н                  | F                                     | Н | 23.0 ± 2.2    | 767.2 ± 16       | 33.4  |
| 16 | Н        | Н                  | Н                                     | F | 60.9 ± 7.16   | 1,335 ± 211      | 21.9  |
| 17 | Н        | Н                  | CH₃                                   | Н | 45.3 ± 0.9    | 587.9 ±<br>18.9  | 13.0  |
| 18 | Н        | $CH_3$             | CH₃                                   | Н | 57.1 ± 11.6   | 1,076 ± 41       | 18.9  |
| 19 | Н        | CH <sub>2</sub> CI | -<br>H <sub>2</sub> CH <sub>2</sub> - | Н | 156.8 ± 4.3   | 1,300 ± 84       | 8.3   |

<sup>a</sup>: SF (selectivity factor): IC<sub>50</sub> values in HUVEC/ IC<sub>50</sub> values in MV4;11.

Considering the bioactivity of the compounds and the diversity of substitution groups, we selected the compounds **1**, **9**, **10**, **15**, and **17** to test their microsome stability (Table 3). Unfortunately, un- or mono-substituted benzothiazolyl compounds showed poor microsome stability. To our delight, **10** demonstrated

good stability in mouse liver microsome (Clint,mouse, 69.6 mL/min/Kg) with longer  $T_{1/2}$  (78.4 min) compared to triapine. Moreover, mouse pharmacokinetic data indicated that this compound had satisfactory ADME properties (AUC: 734 hr•ng/ml,  $T_{1/2}$ : 1.4 hrs and bioavailability: 25%) (Table 4).

Table 3. Microsome stability of triapine derivatives

| compound | T <sub>1/2</sub> (minute) | Clint (mL/min/Kg) |
|----------|---------------------------|-------------------|
| Triapine | 10.5                      | 518.1             |
| 1        | 3.9                       | 1396.1            |
| 9        | 5.6                       | 972.4             |
| 10       | 78.4                      | 69.6              |
| 15       | 3.2                       | 1706.9            |
| 17       | 2.2                       | 2518.8            |

Table 4. Mouse pharmacokinetic parameters of 10

|                         | IV (1 | PO (10   |
|-------------------------|-------|----------|
| T <sub>1/2</sub> (hr)   | 0.59  | 1.40     |
| CI (I/hr/kg)            | 3.39  | <u> </u> |
| C <sub>max</sub> (µg/L) |       | 673      |
| AUC <sub>last</sub>     | 294   | 734      |
| F (%)                   |       | 25.0%    |

10 inhibits the growth of hematopoietic cancer cells in vitro and in vivo. Given the potent activity and its favourable pharmacokinetic properties, we further evaluated the anticancer spectrum of 10. We examined its activity in 25 cancer cell lines and compared it with that of triapine. Generally, 10 was 10 to 100-fold more potent than triapine (Figure 2A, Supplementary Table 1). Moreover, the selectivity of 10 against MV4;11 cells over HUVEC is more than 250 folds, superior to that of triapine (3.6 folds, Table 1 and 2). 10 was more effective at inhibiting the viability of hematopoietic tumor cells than that of solid tumor cells (Figure 2B). Among these cell lines, MV4;11 was the most sensitive cell to the cytotoxic effect of 10. We therefore evaluated 10 in MV4;11 tumor xenograft mouse model. Treatment with 10 orally at a dose of 30 mg/kg significantly inhibited tumor growth (Figure 2C), with no obvious impact on mouse host body weight (Figure 2D). Thus, we have identified a novel triapine derivative 10 that could effectively inhibit the growth of tumor cells, especially leukemia cells.

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Figure 2. The antitumor activity of 10 *in vitro* and *in vivo*. (A) All cell lines were exposed to increasing concentrations of triapine or 10 and the cell viability was tested using a CellTiter-Glo<sup>TM</sup> Luminescent cell viability assay 48 h post-treatment. The IC<sub>50</sub> value was calculated by non-linear regression analysis and a standard sigmoidal dose-response model in the GraphPad Prism program, and the logarithm of mean IC<sub>50</sub> values is presented as a histogram. (B) Summary of

logarithm of mean IC<sub>50</sub> of **10** against solid cancer cells (n=11) compared with hematopoietic cancer cells (n=14). (C) *In vivo* efficacy study of **10**. The tumor volume was calculated based on the width and length of tumors. The mice transplanted with MV4;11 leukemia cells were treated with 30 mg/kg **10** administered orally twice daily, while the control mice were treated with vehicle. \* *P*<0.05, \*\* *P*<0.01. (D) Body weight of mice during 14 days of **10** treatments.

**10** induces mitochondrial ROS-dependent cell death. We next analyzed the molecular mechanism underlying the antitumor activity of **10**. Treatment of **10** led to profound G1 arrest in MV4;11 cells after 24 h and cell death after 48 h (Figure S1A). The cell death was not rescued by Z-VAD or/and Nec-1, suggesting that the cells did not undergo apoptosis or necroptosis (Figure S1B). We then asked whether **10** triggers ferroptotic cell death in MV4;11 cells. Ferrostatin-1, which has been reported to block ferroptosis,<sup>41</sup> failed to inhibit **10**-induced cell death either (Figure S1C). Because ROS plays an important role in a variety of cell deaths induced by iron chelators,<sup>16, 26</sup> we next tested whether ROS contributes to the cytotoxic activity of **10**. MV4;11 cells were pretreated with various ROS

scavengers prior to the addition of 10. Only N-acetyl-L-cysteine (NAC), but not butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) or  $\alpha$ -tocopherol ( $\alpha$ -TOC), significantly attenuated **10**-induced cell death (Figure 3A). Among the ROS scavengers, only NAC structurally contains a sulfhydryl group, making us question whether reduced glutathione (GSH) could block the cell death induced by 10. Indeed, treatment with GSH was sufficient to restore the cell viability after 10 addition (Figure 3B). We also found that the ratio of GSH/GSSG, an essential indicator of cellular oxidative stress,<sup>42</sup> was decreased in **10**-treated MV4;11 cells (Figure 3C), suggesting that redox homeostasis was disturbed by the treatment of 10. We found that 10 treatment elevated the levels of mitochondrial ROS, but not intracellular ROS in MV4;11 cells (Figure 3D). Pretreatment with 5 mM NAC 1 h before 10 addition led to almost complete inhibition of mitochondrial ROS generation (Figure 3E). Interestingly, triapine treatment did not increase the levels of mitochondrial ROS (Figure 3F), suggesting that 10 and triapine induce cell death through different mechanism.



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| Figure 3. 10 selectively induces mitochondrial oxidative stress in MV4;11 cells.                     |
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| (A) MV4;11 cells were pretreated with 100 $\mu M$ BHA, 100 $\mu M$ BHT, 10 $\mu M$ $\alpha \text{-}$ |
| TOC, 0.5 mM NAC or 5 mM NAC and incubated with 10, and the cell viability                            |
| was measured with the CellTiter-Glo <sup>TM</sup> Luminescent cell viability assay. (B)              |
| MV4;11 cells were pretreated with 5 mM GSH and incubated with 10. The cell                           |
| viability was measured. (C) Measurement of the ratio of reduced glutathione (GSH)                    |
| to oxidized glutathione (GSSG) in MV4;11 cells treated with 10. Cells were treated                   |
| with the GSH/GSSG-Glo <sup>™</sup> assay kit, and the luminescence was measured with                 |
| the SpectraMax i3 microplate reader. Data are representative of two independent                      |
| experiments. *** P< 0.001. (D) MV4;11 cells in 6-well plates were treated with                       |
| 50 nM 10 for 4 or 8 h then stained with CellROX or MitoSOX. Intracellular ROS                        |
| levels or mitochondrial ROS levels were determined with flow cytometry. (E)                          |
| MV4;11 cells in 6-well plates were treated with 50 nM 10 for 4 h in the presence                     |
| or absence of 5 mM NAC. Mitochondrial ROS levels were determined with flow                           |
| cytometry. (F) MV4;11 cells in 6-well plates were treated with 50 nM 10 or 500                       |

nM triapine for 4h then stained with MitoSOX. Mitochondrial ROS levels were determined with flow cytometry.

Cu<sup>2+</sup> cooperates with 10 to inhibit cancer cell survival. It has been shown that the N\*-N\*-S\* terdentate coordination system in triapine allows chelation with transition metal ions.<sup>19, 43</sup> In order to identify the exact chelation partner of **10**, we treated MV4;11 cells with 10 in the presence of various metal ions including Cu<sup>2+</sup>, Ca<sup>2+</sup>, Fe<sup>3+</sup>, Fe<sup>2+</sup>, and Mn<sup>2+</sup>. We found that only incubation with copper could potentiate **10**-induced death (Figure 4A). Incubation with **10** in the presence of Cu<sup>2+</sup> caused an increasing proportion of dead cells (Figure 4B) and decreasing ratio of GSH/GSSG (Figure 4C) compared to the treatment of 10 alone. We next examined the potential synergy of **10** with  $Cu^{2+}$  on inhibiting MV4;11 cell survival. The combination index (CI) values showed the strong synergistic effect of Cu<sup>2+</sup> and **10** (Figure 4D). Interestingly, copper exhibited antagonistic effect on triapine's activity (Figure 4D), suggesting that although 10 and triapine are structurally related, they utilize different metal ions to execute anti-tumor activity. Then we evaluated the chelation ability of **10** and triapine with Cu or Fe by UV-vis







Figure 4. Cu<sup>2+</sup> cooperates with 10 to inhibit cancer cell survival. (A) MV4;11

cells were pretreated with 50 µM Cu<sup>2+</sup>, Ca<sup>2+</sup>, Fe<sup>3+</sup>, Fe<sup>2+</sup> or Mn<sup>2+</sup> and incubated with indicated concentrations of 10 starting from 100 nM with 3-fold dilution series. The cell viability was measured with the CellTiter-Glo<sup>™</sup> Luminescent cell viability assay. (B) Cells were grown in 12-well plates and treated with 10 for 24 h in the presence or absence of Cu<sup>2+</sup>. Apoptosis was detected by flow cytometry using Annexin V-FITC and propidium iodide (PI) double staining. (C) Measurement of the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) in MV4;11 cells treated with 10 in the absence or presence of Cu<sup>2+</sup>. Cells were treated with the GSH/GSSG-Glo<sup>™</sup> assay kit, and the luminescence was measured with the SpectraMax i3 microplate reader. Data are representative of two independent experiments. \* P< 0.05, \*\*\* P< 0.001. (D) Analysis of combination effects between 10 or triapine with Cu<sup>2+</sup> for MV4;11 cells. Combination index analysis was carried out using the Calcusyn software. The logarithm of CI values is shown. Green indicates synergism while red indicates antagonism.

10 triggers copper-dependent cell stress and death. We next asked if 10induced cell death is dependent on intracellular copper. We found that cell death induced by 10 treatment was suppressed by co-treatment with copper chelator bathocuproine disulfonate (BCS) or ammonium tetrathiomolybdate (TM),<sup>24,44</sup> but not iron chelator deferoxamine (DFO) (Figures 5A). Consistently, 10-induced mitochondrial ROS production was attenuated by BCS treatment, indicating that intracellular copper is indispensable for the cytotoxic activity of **10** (Figures 5B). Then we investigated whether copper homeostasis is disturbed by 10. We employed inductively coupled plasma mass spectrometry (ICP-MS) to measure the total intracellular copper content. Addition of 10 modestly increased intracellular copper content, suggesting that the copper homeostasis in the cells were altered by **10** (Figures 5C). Cotreatment of **10** with Cu<sup>2+</sup> significantly increased intracellular copper content, whereas triapine had minimal effects on intracellular copper abundance (Figure 5C). Copper chelator BCS completely prevented the increase in copper content after exposure to **10** and Cu<sup>2+</sup> (Figures

5C). These data suggested that 10 promoted cell death by inducing intracellular

copper accumulation and copper-dependent ROS production.





indicated concentrations of 10 starting from 5 µ M with 3-fold dilution series, and

the cell viability was measured. (**B**) MV4;11 cells in 6-well plates were treated with 50 nM compound **10** for 4 h in the presence or absence of 200  $\mu$ M BCS. Mitochondrial ROS levels were determined with flow cytometry. (**C**) The addition of **10** and Cu<sup>2+</sup> lead to an increasing in total cellular copper for 12 h, but BCS treatment can restore the total copper content. Data are representative of two independent experiments. \* *P*< 0.05, \*\* *P*<0.01, \*\*\* *P*< 0.001.

**10** increases TXNIP expression and induces the activation of JNK and p38 MAPK. GSH/GSSH ratio was reduced in **10**-treated cells, suggesting that the thiol redox was disrupted by **10** treatment (Figure 3C). Cellular thiols are susceptible to several oxidants, and the thioredoxin (TRX) is critical for maintaining intracellular thiol redox balance.<sup>29, 32</sup> Indeed, treatment with **10** decreased TRX activity (Figure 6A). Previous studies have suggested that TXNIP can bind and inhibit TRX, thus activating JNK and p38 MAPK pathway. Consistently, we observed an obvious increase in both mRNA and protein levels of TXNIP induced by **10** treatment in MV4;11 cells (Figures 6B, 6C). Consequently, JNK and p38

were phosphorylated following **10** treatment (Figure 6C), which may potentiate the cell oxidative stress effect of **10**. However, triapine had no effects on TXNIP level and the activation of JNK and p38 (Figure S3A).



Figure 6. 10 increases TXNIP expression and induces the activation of JNK and p38 MAPK. (A) TRX activity in MV4;11 cells treated with DMSO or 50 nM 10 was measured at 412 nm. Data are representative of three independent experiments. \*\* P< 0.01. (B) MV4;11 cells were treated with 10 for 24 h, and

then qPCR assay using two pairs of TXNIP primers was performed. \* P < 0.05, \*\*\* P < 0.001. (**C**) MV4;11 cells were treated with 50 nM **10** w/o NAC for 24 h. Whole-cell lysates were prepared, and the expression of TXNIP and the phosphorylated form of JNK and p38 were determined by western blotting. GAPDH served as a loading control.

10 induces mitochondrial dysfunction. Saxena et al. have described that mitochondrial shutting of TXNIP, under oxidative stress, affected mitochondrial dysfunction and oxidation of mitochondrial DNA (mtDNA).<sup>45</sup> As the loss of mitochondrial membrane potential (MMP,  $\Delta \Psi m$ ) is associated with the production of mitochondrial ROS, we examined the effect of 10 treatment on MMP. The treatment of **10** resulted in loss of MMP detected by flow cytometry using cationic dye JC-1 (Figure 7A), whereas triapine treatment had no impact (Figure S3B). mtDNA is physically associated with the inner mitochondrial membrane, and elevated ROS levels could lead to mtDNA damage.<sup>46</sup> In MV4;11 cells, we detected a significant decrease in mtDNA copy number following treatment with **10** (Figure 7B), but with no decrease of mitochondrial mass (Figure 7C).

Furthermore, **10** markedly reduced oxygen consumption and impaired respiration on mitochondrial function (Figures 7D). The morphologies of mitochondria in MV4;11 cells were examined by transmission electron microscope (TEM). 24 hours after the addition of **10**, mitochondria with a clear swelling and less number of cristae or deformed cristae were observed (Figure 7E). These results show that **10** treatment can reduce mitochondrial oxidative phosphorylation (OXPHOS) and affect mitochondrial morphology and function.



Figure 7. 10 triggers the mitochondrial dysfunction pathway. (A) 10 induced the collapse of  $\Delta \Psi m$ . The cells stained with JC-1, followed by FACS analysis. Data are representative of two independent experiments. \*\* P< 0.01. (B) Mitochondrial DNA copy number was assessed as the relative amplification of MT-ND2 (mtDNA

> gene) and HPRT (nDNA gene). Data are representative of three independent experiments. **\*\*** P< 0.01. (C) Flow cytometric analysis of mitochondrial mass in **10**-untreated and -treated MV4;11 cells with MitoTracker Deep Red dye. (D) Oxygen consumption was measured in MV4;11 cells treated with DMSO (red line) and with **10** (blue line) for 24 h. Basal ORC (pmol of O<sub>2</sub>/min) and maximum reserve capacity were analyzed using XF software. The trace represented the mean ± SEM of four independent experiments and a schematic illustration of respiratory parameters. **\*** P< 0.05, **\*\*** P< 0.01, **\*\*\*** P< 0.001. (E) Representative transmission electron microscopy images of MV4;11 cells. Yellow arrowheads indicate abnormal mitochondria.

#### **DISCUSSION AND CONCLUSION**

Findings from clinical trials have shown that triapine administered in combination with chemo- or radiotherapy is effective against limited human cancer types (such as hematologic malignancies and cervical cancer), but not in many others (such as recurrent head and neck squamous cell carcinoma and advanced pancreatic cancer),<sup>9, 47-50</sup> probably due to the weak potency of triapine. In this study, we

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identified a triapine derivative, compound IC25 (**10**), with more potent activity against cancer cells. We found that hematopoietic cancer cells exhibited greater sensitivity to **10** than solid tumor cells. Importantly, **10** showed antitumor activity *in vivo* via oral administration, demonstrating pharmacokinetic advantage over triapine which is administrated intravenously.

Although **10** is structurally derived from triapine, it executes anti-tumor activity via a different mechanism as triapine does. **10** cooperates with copper to induce copper-dependent mitochondrial ROS production. In contrast, triapine barely induces mitochondrial ROS and has no synergy with copper, instead, copper antagonizes triapine's anti-tumor activity. Interestingly, we observed that addition of Fe, Ca or Mn ion slightly inhibited the cytotoxic activity of **10** (Figure 4A), suggesting that these ions may compete with intracellular copper to bind to **10** and inactivate its anti-tumor activity.

**10** induces copper-dependent mitochondrial ROS accumulation and mitochondrial damage in leukemia cells. Previous study has reported that acute myeloid leukemia (AML) blasts displayed significantly lower mitochondrial ROS

levels and mitochondrial gene expression than normal cells.<sup>51</sup> Some reports indicated that copper levels both in serum and tumor are elevated in cancer patients,<sup>21, 52</sup> which may account for the selectivity of **10** against leukemia cells over solid tumors and normal cells.

Taken together, **10** induced copper-dependent mitochondrial ROS accumulation in hematopoietic tumor cells, leading to mitochondrial dysfunction and the phosphorylation of JNK and p38 MAPK through TXNIP-TRX pathway, which contributes to the antitumor activity of **10**. We herein provide a potential approach for increasing mitochondrial ROS with copper, which may be helpful to target aggressive blood tumors with poor prognosis.

#### EXPERIMENTAL SECTION

**General Methods for Chemistry.** All reagents and starting materials were obtained from commercial suppliers and used without further purification unless otherwise stated. Reaction progress was monitored by thin layer chromatography (TLC) on preloaded silica gel 60 F254 plates. Visualization was achieved with UV light and iodine vapor. All reactions involving oxygen- or moisture-sensitive compounds

were carried out under a dry N2 atmosphere. Yields were of purified product and were not optimized. <sup>1</sup>H NMR, <sup>13</sup>C NMR were recorded on Bruker AM-400 spectrometer in the corresponding solvent. <sup>1</sup>H NMR spectra were referenced to the residual solvent peaks as internal standards (7.26 ppm for CDCl<sub>3</sub>, 2.50 ppm for DMSO-d<sub>6</sub>, and 3.34 ppm for CD<sub>3</sub>OD). <sup>13</sup>C NMR spectra were referenced to the residual solvent peaks as internal standards (39.52 ppm for DMSO- $d_6$ ), NMR data were recorded as follows: multiplicity (s = singlet, d = doublet, t =triplet, m = multiplet or unresolved, coupling constant (solid) in Hz, integration). Mass spectra were determined on an Agilent 5973N MSD (EI), Shimadzu LCMS-2010EV (ESI) mass spectrometer or Agilent G6100 LC/MSD (ESI) single Quand mass spectrometer and lonSpec HiResMALDI. High resolution mass spectra were recorded on Waters Micromass GCT Premier (EI), Bruker Daltonics, Inc. APEXIII 7.0 TESLA FTMS (ESI) mass spectrometers and lonSpec 4.7 Tesla FTMS (MALDI). The purity was determined by high performance liquid chromatography (HPLC). Purity of all final compounds was 95% or higher. The instrument was

an Agilent Technologies 6120 LC/MS system. The column was a Phenomenex Luna C18, 100 A, 2.0 50 mm, 5 μm.

## General Procedure A : The synthesis of different substituted 2-amino benzothiazole.

A mixture of different substituted aniline (1 mmol, 1 eq) and potassium thiocyanate (389 mg, 4 mmol, 4 eq) in 1 mL glacial acetic acid was cooled in an ice bath and stirred for 10- 20 min, then bromine (1 mmol, 1 eq) in 1ml glacial acetic acid was added dropwise at such a rate to keep the temperature below 10 °C throughout the addition. The reaction mixture was stirred at room temperature for 3 h. After completion of reaction (as monitored by TLC), the reaction mixture was basified to pH 11.0 with ammonia solution (NH<sub>4</sub>OH) and the resulting precipitate was filtered, washed with water and dried to get the desired product.<sup>37</sup>

**General Procedure B: The synthesis of different substituted 2-hydrazinyl benzo thiazole.** To a solution of 80% hydrazine hydrate (0.37 ml, 6 mmol, 6 eq) in ethylene glycol at 0 °C, conc. HCI (0.29 ml, 3.4 mmol, 3.4 eq) was added dropwise with stirring, followed by the addition of different substituted 2-amino

benzothiazole (1.0 mmol, 1.0 eq). The reaction mixture was refluxed for 5 h and then cooled to the room temperature. Water was added to the reaction liquid, the resulting solid formed was filtered, washed with water, and recrystallized from ethanol to afford the compounds. <sup>38</sup>

General Procedure C: The synthesis of 7-substituted 2-chloro-benzo[d]thiazole. A solution of different substituted 2-fluoroaniline (11.6 mmol, 1.0 eq), potassium O-ethyl dithiocarbonate (4.1 g, 25.6 mmol, 2.2 eq) in 7.5 mL anhydrous DMF was heated at 120 °C for 4 hours under nitrogen. After completion of reaction (as monitored by TLC), the reaction mixture was cooled to room temperature, and diluted with H<sub>2</sub>O (15mL) and 1 N HCl solution (20 mL) to induce precipitation. Stirring was continued for 30 minutes. The solid precipitate was collected by filtration, and rinsed with water. The wet filter cake was dissolved in 250 mL EtOAc, and dried over Na<sub>2</sub>SO<sub>4</sub>. EtOAc was removed by rotary evaporation, and the residue was dried in vacuo to afford 7-substituted 2-mercaptobenzothiazole. Without further purification, to 7-substituted 2-mercaptobenzothiazole (9.8 mmol) cooled in an ice-water bath, was added 4.9 mL SO<sub>2</sub>Cl<sub>2</sub> at 0 °C under nitrogen,
and the suspension was stirred at room temperature for 2 h. After completion of reaction (as monitored by TLC), the reaction mixture was poured onto ice water with stirring. Precipitation was formed, and stirring was continued for 2 h. The solid precipitate was collected by filtration, and rinsed with water. The solid was dried in vacuo to afford desired product.<sup>39</sup>

# General Procedure D: The synthesis of 7-substituted 2-

**hydrazinylbenzo[d]thiazole.** 80% hydrazine hydrate (0.67 ml, 10.8 mmol, 4 eq) was added to a solution of 7-substituted 2-chloro-benzo[d]thiazole (2.7 mmol, 1.0 eq) in 10.8 mL EtOH, then the mixture was heated at 70 °C for 5 h. After completion of reaction (as monitored by TLC), the reaction mixture was cooled to room temperature, EtOH was removed by rotary evaporation and diluted with 20ml H<sub>2</sub>O. Precipitation was formed, the solid precipitate was collected by filtration, and rinsed with water. The solid was dried in vacuo to afford desired product. <sup>39</sup>

**General Procedure E: The synthesis of compounds 1-19.** A mixture of 6,7dihydroquinolin-8(5H)-one or other compounds with a ketone group (1.0 mmol,

1.0 eq) and different substituted 2-hydrazinyl benzothiazole (1.2 mmol, 1.2 eq) was dissolved in 8ml methanol, then acetic acid (0.06 ml, 1.0 mmol, 1.0 eq) was added. The reaction mixture was refluxed for 5 h. After completion of reaction (as monitored by TLC), the excess solvent was evaporated under reduced pressure. The resultant solid was purified by column chromatography over silica gel (MeOH /  $CH_2Cl_2$  as the eluent) to afford the compounds.

(E)-2-(2-(6,7-dihydroquinolin-8(5H)-ylidene)hydrazinyl)benzo[d]thiazole (1). The title compound was prepared from 6,7-dihydroquinolin-8(5H)-one and 2-hydrazinyl benzothiazole following the general procedure E (236 mg, 80% yield). <sup>1</sup>H NMR (400 MHz, Chloroform-d) δ 8.65 (q, J = 4.3 Hz, 1H), 7.77 - 7.57 (m, 2H), 7.51 (d, J = 7.7 Hz, 1H), 7.39 - 7.28 (m, 2H), 7.12 (t, J = 9.2 Hz, 1H), 2.99 - 2.78 (m, 4H), 2.03 - 1.90 (m, 2H).<sup>13</sup>C NMR (100 MHz, Chloroform-d) δ 126.46, 123.26, 122.50, 122.48, 121.53, 28.55, 25.51, 20.58. HRMS-ESI: calcd. For C<sub>16</sub>H<sub>15</sub>N<sub>4</sub>S [M + H]<sup>+</sup>, 295.1012; found, 295.1018. HPLC purity: 98.4%.

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| (E)-8-(2-(1H-benzo[d]imidazol-2-yl)hydrazono)-5,6,7,8-tetrahydroquinoline (2).  |
|---|
| The title compound was prepared from 6,7-dihydroquinolin-8(5H)-one and 2-   |
| hydrazinyl-1H-benzo[d]imidazole following the general procedure E (208 mg, 75%  |
| yield). <sup>1</sup> H NMR (400 MHz, Chloroform-d) $\delta$ 8.52 (d, J = 4.7 Hz, 1H), 7.59 (d, J                                    |
| = 8.2 Hz, 1H), 7.38 - 7.33 (m, 2H), 7.25 - 7.21 (m, 1H), 7.16 - 7.11 (m, 2H),   |
| 2.89 - 2.79 (m, 4H), 1.99 (p, J = 6.4 Hz, 2H). $^{13}$ C NMR (100 MHz, Methanol-d <sub>4</sub> )                                    |
| δ 155.77, 150.77, 148.34, 146.16, 139.27, 137.21, 124.74, 122.28, 113.29, 29.80,  |
| 26.77, 22.10. HRMS-ESI: calcd. for C <sub>16</sub> H <sub>16</sub> N <sub>5</sub> [M + H] <sup>+</sup> : 278.1400, found: 278.1398. |
| HPLC purity: 98.5%.   |

# (E)-2-(2-(5,6-dihydro-7H-cyclopenta[b]pyridin-7-

ylidene)hydrazinyl)benzo[d]thiazole (3). The title compound was prepared from 5,6-dihydro-7H-cyclopenta[b] pyridin-7-one and 2-hydrazinyl-1H-benzo[d]imidazole following the general procedure E (224 mg, 80% yield). <sup>1</sup>H NMR (400 MHz, Chloroform-d)  $\delta$  8.66 (d, J = 5.4 Hz, 1H), 7.71 - 7.64 (m, 2H), 7.56 (d, J = 8.1 Hz, 1H), 7.34 (t, J = 7.7 Hz, 1H), 7.26 - 7.23 (m, 1H), 7.21 - 7.15 (m, 1H), 3.20 - 3.11 (m, 2H), 2.96 - 2.88 (m, 2H). <sup>13</sup>C NMR (100 MHz, DMSO-d<sub>6</sub>)  $\delta$  168.45,

156.06, 149.65, 143.10, 134.20, 126.37, 124.36, 122.03, 27.23, 26.04. HRMS-ESI: calcd. for  $C_{15}H_{13}N_4S$  [M + H]<sup>+</sup>: 281.0855, found: 281.0851. HPLC purity: 98.7%.

(E)-2-(2-(3,4-dihydronaphthalen-1(2H)-ylidene)hydrazinyl)benzo[d]thiazole (4). The title compound was prepared from 3,4-dihydronaphthalen-1(2H)-one and 2-hydrazinylbenzo[d]thiazole following the general procedure E (228 mg, 78% yield). <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>)  $\delta$  11.62 (s, 1H), 8.11 - 8.00 (m, 1H), 7.73 (d, J = 7.8 Hz, 1H), 7.36 (s, 1H), 7.31 - 7.23 (m, 3H), 7.20 (dt, J = 5.7, 3.1 Hz, 1H), 7.09 (td, J = 7.6, 1.2 Hz, 1H), 2.77 (t, J = 6.3 Hz, 4H), 1.85 (p, J = 6.4 Hz, 2H).<sup>13</sup>C NMR (100 MHz, DMSO-d<sub>6</sub>)  $\delta$  168.54, 139.93, 132.82, 129.19, 129.11, 126.71, 126.37, 124.45, 122.13, 121.90, 29.49, 26.89, 21.95. HRMS-ESI: calcd. for C<sub>17</sub>H<sub>16</sub>N<sub>3</sub>S [M + H]<sup>+</sup>:294.1059, found: 294.1059. HPLC purity: 99.3%.

(E)-4-(2-(benzo[d]thiazol-2-yl)hydrazono)-3,4-dihydro-2H-pyrano[3,2-b]pyridine (5). The title compound was prepared from 2,3-dihydro-4H-pyrano[3,2-b]pyridine-4-one and 2-hydrazinylbenzo[d]thiazole following the general procedure E (213 mg, 72% yield). <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>)  $\delta$  11.81 (s, 1H), 8.32 (dd, J = 4.2, 1.7 Hz,

| 1H), 7.72 (d, J = 7.0 Hz, 1H), 7.44 - 7.22 (m, 4H), 7.10 (t, J = 7.6 Hz, 1H), 4.33  |
|---|
| (t, J = 6.1 Hz, 2H), 3.08 (t, J = 6.1 Hz, 2H). <sup>13</sup> C NMR (100 MHz, DMSO-d <sub>6</sub> ) $\delta$                   |
| 168.91, 154.02, 143.50, 139.02, 126.46, 125.48, 125.38, 122.33, 122.08, 65.51,  |
| 27.14. HRMS-ESI: calcd. for C <sub>15</sub> H <sub>13</sub> N <sub>4</sub> OS [M + H] <sup>+</sup> :297.0805, found:297.0804. |
| HPLC purity: 98.5%.   |

# (6). The title compound was prepared from 2,3-dihydro-4H-pyrano[3,2-b]pyridin-4-

(E)-2-(2-(6,7-dihydroquinolin-8(5H)-ylidene)-1-methylhydrazinyl)benzo[d]thiazole

one and 2-(1-methylhydrazinyl)benzo[d]thiazole (**6a**) following the general procedure E (169 mg, 55% yield). <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>)  $\delta$  8.57 (dd, J = 4.6, 1.7 Hz, 1H), 7.80 (d, J = 7.7 Hz, 1H), 7.69 (dt, J = 8.0, 1.8 Hz, 1H), 7.61 (dd, J = 8.0, 1.1 Hz, 1H), 7.40 (dd, J = 7.7, 4.5 Hz, 1H), 7.32 (ddd, J = 8.3, 7.3, 1.3 Hz, 1H), 7.14 (td, J = 7.6, 1.2 Hz, 1H), 3.58 (s, 3H), 2.97 (t, J = 6.4 Hz, 2H), 2.90 (t, J = 6.1 Hz, 2H), 1.97 - 1.87 (m, 2H). <sup>13</sup>C NMR (100 MHz, DMSO-d<sub>6</sub>)  $\delta$  172.01, 160.39, 152.07, 148.62, 148.05, 137.03, 136.66, 132.13, 125.67, 124.73, 121.73, 121.34, 119.51, 41.32, 29.72, 28.18, 22.01. HRMS-ESI: calcd. for C<sub>17</sub>H<sub>17</sub>N<sub>4</sub>S [M + H]<sup>+</sup>:309.1168, found:309.1168. HPLC purity: 97.7%.

| 2-(1-methylhydrazinyl)benzo[d]thiazole (6a). To a colorless solution of 2-                          |
|---|
| chlorobenzothiazole (350.2 mg, 2.1 mmol) in absolute EtOH (4 mL), a fivefold                        |
| excess of methylhydrazine (482.4 mg, 549.4 $\mu\text{L},$ 10.3 mmol) was added under                |
| an $N_2$ atmosphere. The mixture was stirred for 6 h under reflux. After completion                 |
| of reaction (as monitored by TLC), reaction mixture was cooled to -12 $^\circ\text{C},$ the         |
| final product precipitated as white needles and was collected by filtration under                   |
| reduced pressure. <sup>40</sup> Yield: 310 mg, 83.7 %. $^1\text{H}$ NMR (400 MHz, DMSO-d6) $\delta$ |
| 7.66 (dd, J = 7.8, 1.4 Hz, 1H), 7.36 (dd, J = 8.0, 1.1 Hz, 1H), 7.20 (ddd, J =                      |
| 8.1, 7.3, 1.3 Hz, 1H), 6.98 (td, J = 7.5, 1.2 Hz, 1H), 5.39 (s, 2H), 3.32 (s, 3H).                  |
| (E)-2-(6,7-dihydroquinolin-8(5H)-ylidene)-1-(4-methoxybenzo[d]thiazol-2-                            |

yl)hydrazin-1-ium formate (7). The title compound was prepared from 3,4dihydronaphthalen-1(2H)-one and 2-hydrazinyl-4-methoxybenzo[d]thiazole following the general procedure E (260 mg, 80% yield). <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>)  $\delta$ 8.53 (dd, J = 4.6, 1.7 Hz, 1H), 7.61 (dd, J = 7.7, 1.7 Hz, 1H), 7.38 (dd, J = 7.9, 1.0 Hz, 1H), 7.27 (dd, J = 7.6, 4.6 Hz, 1H), 7.08 (t, J = 8.0 Hz, 1H), 6.92 (dd, J = 8.1, 1.0 Hz, 1H), 3.88 (s, 3H), 2.85 - 2.75 (m, 4H), 1.87 (p, J = 6.5

> Hz, 2H).<sup>13</sup>C NMR (100 MHz, DMSO-d<sub>6</sub>) δ 167.59, 150.36, 149.48, 147.93, 146.83, 136.35, 135.26, 131.24, 123.16, 122.41, 113.72, 107.99, 55.77, 28.36, 26.86, 20.87. HRMS-ESI: calcd. for C<sub>17</sub>H<sub>17</sub>N<sub>4</sub>OS [M + H]<sup>+</sup>: 325.1118, found:325.1117. HPLC purity: 98.6%.

(E)-2-(2-(6,7-dihydroquinolin-8(5H)-ylidene)hydrazinyl)-5-

methoxybenzo[d]thiazole (8). The title compound was prepared from 3,4dihydronaphthalen-1(2H)-one and 2-hydrazinyl-5-methoxybenzo[d]thiazole following the general procedure E (223 mg, 69% yield). <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>) δ 11.60 (s, 1H), 8.53 (dd, J = 4.6, 1.7 Hz, 1H), 7.61 (d, J = 6.0 Hz, 2H), 7.27 (dd, J = 7.7, 4.6 Hz, 1H), 6.98 (s, 1H), 6.74 (d, J = 8.7 Hz, 1H), 3.79 (s, 3H), 2.89 - 2.72 (m, 4H), 1.86 (p, J = 6.3 Hz, 2H).<sup>13</sup>C NMR (100 MHz, DMSO-d<sub>6</sub>) δ 169.78, 158.46, 149.56, 147.93, 136.40, 135.39, 123.24, 121.98, 109.43, 55.36, 28.44, 27.08, 20.98. HRMS-ESI: calcd. for C<sub>17</sub>H<sub>17</sub>N<sub>4</sub>OS [M + H]<sup>+</sup>: 325.1118, found:325.1117. HPLC purity: 96.4%.

(E)-2-(2-(6,7-dihydroquinolin-8(5H)-ylidene)hydrazinyl)-6-methoxybenzo[d]thiazole
(9). The title compound was prepared from 6,7-dihydroquinolin-8(5H)-one and 2-

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| hydrazinyl-6-methoxybenzo[d]thiazole following the general procedure E (240 mg,                     |
|---|
| 74% yield). <sup>1</sup> H NMR (400 MHz, Chloroform-d) $\delta$ 8.62 (s, 1H), 7.46 (d, J = 7.8      |
| Hz, 2H), 7.25 - 7.14 (m, 2H), 6.91 (d, J = 8.7 Hz, 1H), 3.84 (s, 3H), 2.77 (t, J                    |
| = 5.9 Hz, 2H), 2.68 (s, 2H), 1.96 - 1.84 (m, 2H). <sup>13</sup> C NMR (100 MHz, Chloroform-d)       |
| δ 167.39, 155.67, 149.48, 148.75, 146.30, 136.50, 135.12, 123.30, 119.71, 113.81,                   |
| 105.53, 55.88, 28.93, 26.29, 21.03. HRMS-ESI: calcd. for $C_{17}H_{17}N_4OS$ [M + H] <sup>+</sup> : |
| 325.1118, found:325.1115. HPLC purity: 98.9%.   |

# (E)-2-(2-(6,7-dihydroquinolin-8(5H)-ylidene)hydrazinyl)-5,6-

dimethoxybenzo[d]thiazole (10). The title compound was prepared from 6,7dihydroquinolin-8(5H)-one and 2-hydrazinyl-5,6-dimethoxybenzo[d]thiazole following the general procedure E (266 mg, 75% yield). <sup>1</sup>H NMR (400 MHz, Chloroformd)  $\delta$  8.61 (d, J = 4.6 Hz, 1H), 7.47 (d, J = 7.6 Hz, 1H), 7.22 - 7.08 (m, 3H), 3.92 (s, 3H), 3.88 (s, 3H), 2.77 (t, J = 5.8 Hz, 2H), 2.69 (s, 2H), 1.94 - 1.85 (m, 2H). <sup>13</sup>C NMR (100MHz, Chloroform-d)  $\delta$ 149.41, 148.82, 146.05, 136.57, 135.11, 123.31, 103.82, 56.50, 56.16, 28.92, 28.23, 21.03. HRMS-ESI: calcd. for C<sub>18</sub>H<sub>19</sub>O<sub>2</sub>N<sub>4</sub>S [M + H]<sup>+</sup>: 355.1223, found:355.1220. HPLC purity: 97.5%.

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| [1,3]dioxolo[4',5':4,5]benzo[1,2-d]thiazole (11). The title compound was prepared   |
|---|
| from 3,4-dihydronaphthalen-1(2H)-one and 6-hydrazinyl-  |
| [1,3]dioxolo[4',5':4,5]benzo[1,2-d]thiazole following the general procedure E (216  |
| mg, 64% yield). <sup>1</sup> H NMR (400 MHz, DMSO-d <sub>6</sub> ) $\delta$ 11.40 (s, 1H), 8.52 (dd, J =  |
| 4.6, 1.7 Hz, 1H), 7.60 (dd, J = 7.7, 1.7 Hz, 1H), 7.41 (s, 1H), 7.26 (dd, J = 7.6,  |
| 4.6 Hz, 1H), 7.09 (s, 1H), 6.03 (s, 2H), 2.79 (t, J = 6.3 Hz, 4H), 1.86 (p, J =   |
| 6.4 Hz, 2H). <sup>13</sup> C NMR (100 MHz, DMSO-d <sub>6</sub> ) $\delta$ 168.24, 149.49, 147.93, 146.56,                                       |
| 143.25, 136.35, 135.18, 123.12, 101.44, 101.06, 28.36, 26.80, 20.85. HRMS-ESI:  |
| calcd. for C <sub>17</sub> H <sub>15</sub> N <sub>4</sub> O <sub>2</sub> S [M + H] <sup>+</sup> :339.0910, found: 339.0910. HPLC purity: 98.4%. |

# (E)-2-(2-(6,7-dihydroquinolin-8(5H)-ylidene)hydrazinyl)-6-

(trifluoromethoxy)benzo[d]thiazole (12). The title compound was prepared from 6,7-dihydroquinolin-8(5H)-one and 2-hydrazinyl-6-(trifluoromethoxy)benzo[d]thiazole following the general procedure E (306 mg, 81% yield). <sup>1</sup>H NMR (400 MHz, Chloroform-d)  $\delta$  8.65 (d, J = 4.6 Hz, 1H), 7.60 - 7.45 (m, 3H), 7.25 - 7.13 (m, 2H), 2.81 (t, J = 6.1 Hz, 2H), 2.70 (t, J = 6.6 Hz, 2H), 1.96 (q, J = 6.3 Hz, 2H).

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<sup>13</sup>C NMR (101 MHz, Chloroform-d)  $\delta$  169.35, 149.84, 148.94, 147.17, 144.26, 136.52, 135.29, 123.64,121.86, 119.79, 119.67, 119.31, 114.54, 28.83, 26.21, 20.98. HRMS-ESI: calcd. for  $C_{17}H_{14}F_3N_4OS$  [M + H]<sup>+</sup>: 379.0835, found:379.0829. HPLC purity: 99.1%.

(E)-2-(2-(6,7-dihydroquinolin-8(5H)-ylidene)hydrazinyl)-4-fluorobenzo[d]thiazole

# (13). The title compound was prepared from 3,4-dihydronaphthalen-1(2H)-one and 4-fluoro-2-hydrazinylbenzo[d]thiazole following the general procedure E (240 mg, 77% yield). <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>) $\delta$ 11.84 (s, 1H), 8.55 (dd, J = 4.6, 1.7 Hz, 1H), 7.65 (dd, J = 18.5, 7.6 Hz, 2H), 7.29 (dd, J = 7.7, 4.6 Hz, 1H), 7.23 - 7.05 (m, 2H), 2.88 - 2.75 (m, 4H), 1.88 (p, J = 6.4 Hz, 2H).<sup>13</sup>C NMR (100 MHz, DMSO-d<sub>6</sub>) $\delta$ 169.45, 149.21, 148.00, 136.45, 135.50, 123.42, 122.24, 122.17, 117.65, 117.61, 111.89, 111.72, 28.28, 26.89, 20.82. HRMS-ESI: calcd. for C<sub>16</sub>H<sub>14</sub>FN<sub>4</sub>S [M + H]<sup>+</sup>: 313.0918 , found: 313.0917. HPLC purity: 98.2%.

# (E)-2-(2-(6,7-dihydroquinolin-8(5H)-ylidene)hydrazinyl)-5-fluorobenzo[d]thiazole

(14). The title compound was prepared from 3,4-dihydronaphthalen-1(2H)-one and 5-fluoro-2-hydrazinylbenzo[d]thiazole following the general procedure E (255 mg,

| 82% yield). <sup>1</sup> H NMR (400 MHz, DMSO-d <sub>6</sub> ) $\delta$ 11.73 (s, 1H), 8.54 (dd, J = 4.6,     |
|---|
| 1.7 Hz, 1H), 7.80 (d, J = 7.7 Hz, 1H), 7.62 (d, J = 7.6 Hz, 1H), 7.29 (dd, J =                                |
| 7.6, 4.6 Hz, 2H), 6.98 (t, J = 9.0 Hz, 1H), 2.92 - 2.72 (m, 4H), 1.87 (p, J = 6.2                             |
| Hz, 2H). <sup>13</sup> C NMR (100 MHz, DMSO-d <sub>6</sub> ) $\delta$ 170.84, 162.48, 160.10, 149.34, 147.98, |
| 136.46, 135.52, 123.40, 122.63, 122.53, 109.13, 108.90, 28.35, 27.01, 20.89.                                  |
| HRMS-ESI: calcd. for $C_{16}H_{14}FN_4S$ [M + H] <sup>+</sup> : 313.0918, found: 313.0917. HPLC               |
| purity: 98.9%.  |

(E)-2-(2-(6,7-dihydroquinolin-8(5H)-ylidene)hydrazinyl)-6-fluorobenzo [d]thiazole (15). The title compound was prepared from 6,7-dihydroquinolin-8(5H)-one and 6fluoro-2-hydrazinylbenzo[d]thiazole following the general procedure E (259 mg, 83% yield). <sup>1</sup>H NMR (400 MHz, Chloroform-d)  $\delta$  8.61 (s, 1H), 7.48 (d, J = 7.7 Hz, 2H), 7.38 (s, 1H), 7.20 (dd, J = 7.7, 4.7 Hz, 1H), 7.05 (t, J = 9.1 Hz, 1H), 2.82 - 2.66 (m, 4H), 1.93 (q, J = 6.4 Hz, 2H). <sup>13</sup>C NMR (100 MHz, Chloroform-d)  $\delta$ 168.57, 159.92,157.50,149.27, 148.76, 136.59, 135.27, 123.54 ,113.81, 113.57, 108.31, 108.03, 28.89, 26.31, 21.01. HRMS-ESI: calcd. for C<sub>16</sub>H<sub>14</sub>FN<sub>4</sub>S [M + H]<sup>+</sup>: 313.0918, found: 313.0914. HPLC purity: 99.7%.

| (E)-2-(2-(6,7-dihydroquinolin-8(5H)-ylidene)hydrazinyl)-7-fluorobenzo[d]thiaz          | ole     |
|--|---------|
| (16). The title compound was prepared from 3,4-dihydronaphthalen-1(2H)-on              | ie and  |
| 7-fluoro-2-hydrazinylbenzo[d]thiazole(16b) following the general procedure E           | E (178  |
| mg, 57% yield). $^1\text{H}$ NMR (400 MHz, DMSO-d_6) $\delta$ 11.87 (s, 1H), 8.56 (do  | 1, J =  |
| 4.5, 1.8 Hz, 1H), 7.63 (dd, J = 7.8, 1.8 Hz, 1H), 7.43 - 7.25 (m, 3H), 7.0             | 1 (t, J |
| = 8.9 Hz, 1H), 2.92 - 2.76 (m, 4H), 1.88 (p, J = 6.3 Hz, 2H). <sup>13</sup> C NMR (100 | MHz,    |
| DMSO-d_6) $\delta$ 157.64, 155.21, 149.23, 148.03, 136.49, 135.63, 127.27, 1           | 23.51,  |
| 107.81, 107.62, 28.35, 27.17, 20.90. HRMS-ESI: calcd. for $C_{16}H_{14}FN_4S$          | [M +    |
| H] <sup>+</sup> :313.0918, found:313.0917. HPLC purity: 96.5%.                         |         |
| (E)-2-(2-(6,7-dihydroquinolin-8(5H)-ylidene)hydrazinyl)-6-methylbenzo[d]thia           | zole    |

(17). The title compound was prepared from 6,7-dihydroquinolin-8(5H)-one and 2hydrazinyl-6-methylbenzo[d]thiazole following the general procedure E (231 mg, 75% yield). <sup>1</sup>H NMR (400 MHz, Chloroform-d)  $\delta$  8.60 (s, 1H), 7.49 - 7.34 (m, 3H), 7.20 - 7.15 (m, 1H), 7.10 (d, J = 8.2 Hz, 1H), 2.79 - 2.67 (m, 4H), 2.40 (s, 3H), 1.89 (q, J = 6.2 Hz, 2H). <sup>13</sup>C NMR (100 MHz, Chloroform-d)  $\delta$  168.52, 149.52,148.51,145.53, 137.53, 136.66, 135.30,132.04, 127.10, 123.33,

121.14,119.22, 28.98, 26.43, 21.25, 21.07. HRMS-ESI: calcd. for C<sub>17</sub>H<sub>17</sub>N<sub>4</sub>S [M + H]<sup>+</sup>: 309.1168, found:309.1165. HPLC purity: 98.6%.

# (E)-2-(2-(6,7-dihydroquinolin-8(5H)-ylidene)hydrazinyl)-5,6-

dimethylbenzo[d]thiazole (18). The title compound was prepared from 6,7dihydroquinolin-8(5H)-one and 2-hydrazinyl-5,6-dimethylbenzo[d]thiazole following the general procedure E (255 mg, 79% yield). <sup>1</sup>H NMR (400 MHz, Chloroform-d)  $\delta$  8.64 (d, J = 4.7 Hz, 1H), 7.46 (d, J = 7.9 Hz, 2H), 7.37 (s, 1H), 7.22 - 7.14 (m, 1H), 2.80 (t, J = 6.0 Hz, 2H), 2.72 (t, J = 6.6 Hz, 2H), 2.33 (s, 6H), 2.01 -1.93 (m, 2H). <sup>13</sup>C NMR (100 MHz, Chloroform-d)  $\delta$  168.72, 149.61, 148.86, 148.66, 146.96, 136.37, 135.03, 134.68, 131.13, 127.88, 123.23, 121.71, 119.53, 28.97, 26.44, 21.10, 20.09, 19.80. HRMS-ESI: calcd. for C<sub>18</sub>H<sub>19</sub>N<sub>4</sub>S [M + H]<sup>+</sup>: 323.1325, found:323.1321. HPLC purity: 99.6%.

(E)-2-(2-(6,7-dihydroquinolin-8(5H)-ylidene)hydrazinyl)-6,7-dihydro-5H-indeno[5,6d]thiazole (19). The title compound was prepared from 6,7-dihydroquinolin-8(5H)one and 2-hydrazinyl-6,7-dihydro-5H-indeno[5,6-d]thiazole following the general

| procedure E (281 mg, 84% yield). <sup>1</sup> H NMR (400 MHz, DMSO-d <sub>6</sub> ) $\delta$ 11.47 (s, |
|--|
| 1H), 8.52 (dd, J = 4.6, 1.8 Hz, 1H), 7.60 (dd, J = 7.7, 1.7 Hz, 2H), 7.47 - 7.24                       |
| (m, 2H), 2.97 - 2.86 (m, 4H), 2.85 - 2.75 (m, 4H), 2.04 (p, J = 7.4 Hz, 2H), 1.86                      |
| (p, J = 6.4 Hz, 2H). $^{13}$ C NMR (100 MHz, DMSO-d <sub>6</sub> ) $\delta$ 168.68, 150.10, 148.36,    |
| 142.47, 138.07, 136.82, 135.71, 123.58, 117.44, 32.72, 32.41, 28.92, 27.46, 26.13,                     |
| 21.45. HRMS-ESI: calcd. for $C_{19}H_{19}N_4S~[M + H]^+$ : 335.1325, found:335.1321.                   |
| HPLC purity: 98.1%.  |

**Cell proliferation assay.** Cell viability was determined with the Celltiter-Glo Luminescent Assay (Promega; Madison, WI, USA). Cells were seeded on 96-well white plates (Corning; Corning, NY, USA) at a density of 5000 cells/well and treated with triapine derivatives with or without metal ion at various concentrations for 48 h, with triplicate samples for each condition. Cells were then exposed to 50 µl of Celltiter-Glo reagent (Promega; Madison, WI, USA) for 10 min at room temperature and the luminescence was measured using the SpectraMax 13 microplate reader (Molecular Devices; Sunnyvale, CA, USA). Control replicates

were cultured in cell culture medium with DMSO and the results were calculated as luminescence of treated replicates/mean luminescense of control replicates.

Combination study. Cell viability was determined with the Celltiter-Glo Luminescent Assay (Promega; Madison, WI, USA). Cells were seeded on 96-well white plates (Corning; Corning, NY, USA) at a density of 5000 cells/well and treated with 10 or triapine with Cu2+ at various concentrations for 48 h, with triplicate samples for each condition. Cells were then exposed to 50 µl of Celltiter-Glo reagent (Promega; Madison, WI, USA) for 10 min at room temperature and the luminescence was measured using the SpectraMax 13 microplate reader (Molecular Devices; Sunnyvale, CA, USA). Survival percentages for three independent biological replicate experiments were averaged and input into CompuSyn software using the Chou - Talay method to extrapolate combination index values.<sup>53</sup> The CIs of <1, 1, and >1 indicate synergism, additive effect, and antagonism, respectively.

**Cell cycle analysis. 10**-treated MV4;11 cells were harvested by centrifugation, washed and kept in 70% chilled ethanol for approximately 12 h, at which point cell pellets were resuspended in propidium iodide with RNase A (Sigma-Aldrich; St. Louis, MO, USA) and diluted with phosphate-buffered saline (PBS) following the manufacturer's instructions. Samples were protected from light and incubated for 30 min before being transferred to FACS tubes. Samples were run on a flow cytometer (FACSCalibur, BD Bioscience; San Jose, CA, USA) and data were analyzed using the ModFit LT software (Verity Software House, Inc.; Topsham, ME, USA).

**Apoptosis analysis.** The Annexin-FITC Apoptosis Detection Kit (Sigma) were used for apoptosis analysis. After resuspended in 500 μl binding buffer, cells were incubated with Annexin V- fluorescein isothiocyanate (FITC, 5 μl) and PI (10 μl). After 10 min incubation, cells were run on a flow cytometer (MoFlo Astrios<sup>EQ</sup>, Beckman Coulter; Brea, CA, USA). All flow cytometric data were analyzed using the software FlowJo version 7.0 (Tree Star, Inc.; Ashland, OR, USA).

**GSH/GSSG ratio assay.** The ratio of GSH to GSSG was measured in the **10**treated and mocked-treated MV4;11 cells. Cells were treated using the GSH/GSSG-Glo<sup>™</sup> Assay Kit (Promega; Madison, WI, USA) following the manufacturer's instructions, and the luminescence was measured with a SpectraMax I3 microplate reader (Molecular Devices; Sunnyvale, CA, USA).

UV-visible spectroscopic titration. The association constants of triapine or 10 and Cu<sup>2+</sup>, Fe<sup>2+</sup> and Fe<sup>3+</sup> were determined by spectrophotometric titration in a 0.1 M KCl in 30%(w/w) DMSO/H<sub>2</sub>O solvent mixture at room temperature (25 °C) with a CARY 100 Scan UV-Vis Spectrophotometer. <sup>54-55</sup> The stock triapine or 10 was diluted to 50  $\mu$  M for 3 ml in a 4 cm quartz cuvette and titrated with 3  $\mu$  l increments of 5 mM of CuCl<sub>2</sub> solution, mixing and waiting 1 min for complex formation. The UV-Vis spectrum from 200 to 800 nm was recorded after each addition by ChemStation Software. Data analysis was carried out using nonlinear regression program SigmaPlot 12.5. If nL + M = MLn, L represents compound, M represents metal ion, then K =  $\frac{[ML_n]}{[L]^n[M]}$ .

Mitochondrial and intracellular reactive oxygen species (ROS) levels. Mitochondrial ROS levels were measured by the MitoSOX<sup>™</sup> Red fluorescence assay (Life Technologies; Foster City, CA, USA). Cells were incubated in presence of 5 µM MitoSOX<sup>™</sup> Red reagent for 10 min at 37 °C. Relative MitoSox Red fluorescence was measured using a flow cytometer (FACSCalibur, BD Bioscience). Intracellular ROS levels were measured by the CellROX Deep Red fluorescence (Life Technologies; Foster City, CA, USA). 10-treated MV4;11 cells were incubated in the presence of 5 µM CellROX Deep Red reagent for 10 min at 37 °C. Relative fluorescence was measured using a flow cytometer (FACSCalibur, BD Bioscience; San Jose, CA, USA). All flow cytometric data were analyzed using the software FlowJo version 7.0 (Tree Star, Inc.; Ashland, OR, USA). Mitochondrial membrane potential (MMP) measurement. The effect of drug treatment on MMP was analyzed using MitoProbe<sup>™</sup> JC-1 Assay kit (Life Technologies; Foster City, CA, USA). The collapse in the electrochemical gradient across the mitochondrial membrane was measured using a fluorescent cationic dye 5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzamidazolo-carbocyanin iodide, also

known as JC-1. This dye exhibits potential dependent accumulation in the mitochondrial matrix. Cells were incubated with 2  $\mu$ M JC-1 for 15 min at 37 °C. We calculated MMP as a ratio for green positive and red negative cells using a flow cytometer (FACSCalibur, BD Bioscience; San Jose, CA, USA). All flow cytometric data were analyzed using the software FlowJo version 7.0 (Tree Star, Inc.; Ashland, OR, USA).

**Mitochondrial mass measurement.** The effect of drug treatment on mitochondrial mass was analyzed using Mitotracker Deep Red (Life Technologies; Foster City, CA, USA). Cells were incubated in presence of 100 nM Mitotracker Deep Red reagent for 30 min at 37 °C. Relative Mitotracker Deep Red fluorescence was measured using a flow cytometer (FACSCalibur, BD Bioscience).

**Mitochondrial stress test.** The oxygen consumption rate (OCR) was measured using a Seahorse XF96 Flux Analyzer with XF Cell Mito Stress Test Kit (Seahorse Bioscience, Inc.; Billerica, MA, USA) according to the manufacturer's instructions. Briefly, cells were plated in XF96 Cell Culture Microplates at a density of 4 × 10<sup>4</sup> cells per well using Poly-L-Lysine (Sigma-Aldrich; St. Louis, MO, USA).

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| Following incubation in XF Base medium supplemented with 10 mM glucose, 2                  |
|--|
| mM L-glutamine and 1 mM sodium pyruvate at 37 $^\circ\text{C}$ (non-CO_2 incubator) for 1  |
| h, ORC was measured at the resting stage (basal respiration) and in response               |
| to oligomycin (1 $\mu$ M, mitochondrial ATP production), mitochondrial uncoupler           |
| FCCP (2 $\mu$ M, maximal respiration), and respiratory chain inhibitor antimycin and       |
| rotenone (0.5 $\mu M$ ). Basal ORC (pmol of $O_2/\text{min})$ and maximum reserve capacity |
| were analyzed using XF software. Spare respiratory capacity was defined as the             |
| difference between maximal respiration and basal respiration. Calculated                   |
| respiratory parameters: Basal (base line OCR minus rotenone plus AA OCR),                  |
| ATP Production (base line OCR minus oligomycin OCR), Spare Capacity (FCCP                  |
| OCR minus base line OCR), Proton Leak (oligomycin OCR minus rotenone plus                  |
| AA OCR).   |

**Transmission electron microscopy (TEM).** MV4;11 cell pellets were collected, fixed with 2.5% glutaraldehyde, post-fixed in 1% OsO4 for 2 h, dehydrated in graded-ethanol series, and embedded in epoxy resin. The embedded cells were sectioned into ultrathin slices, stained by uranyl acetate solution and lead ccitrate,

and then observed with a transmission electron microscopy Quanta 250 (FEI, Eindhoven, The Netherlands).

TRX activity assay. The thioredoxin (Trx) "insulin-reducing assay" was carried out with modifications.<sup>56</sup> MV4;11 cells were grown for 24 h in complete medium containing 50 nM 10 or DMSO. Cells were harvested and lysed. Then, the protein concentration in the cell lysate samples was adjusted to 30 µg of protein in a final volume of 34  $\mu$ L and preincubated with 1  $\mu$ L of 2 mM DTT in 50mM HEPES, pH 7.6, 1 mM EDTA, 1 mg/mL BSA at 37 °C in a waterbath for 15 minutes. The samples were then incubated with a 20  $\mu$ L reaction mixture (200  $\mu$ L of 1 M HEPES pH 7.6, 40 µL of 0.2 M EDTA, 40 µL of NADPH 40 mg/mL, 500 µL of insulin 4.5 mg/mL, and 0.5 U of Trx reductase) at 37 °C for 20 min. The reaction was terminated by the addition of 250 µL of 6 M guanidine HCl, 1 mM DTNB in 0.2 M Tris-HCl pH 8.0). Absorbance was measured at 412 nm in a SpectraMax 13 microplate reader (Molecular Devices; Sunnyvale, CA, USA). All the reagents were purchased from Sigma-Aldrich.

Total Copper Content in MV4;11 Cells by ICP-MS detection. The total copper content of cells incubated for 12 h with media having 50 nM 10 were determined using ICP-mass spectroscopy (Thermo Scientific<sup>TM</sup>, iCAP Q). We collected 9 ×  $10^6$  cells for total Cu uptake experiments and acidified with 400 µl 70% nitric acid. The samples were heated in a microwave digestion unit (Cem, Mars X-Press, North Carolina, USA) fitted with a control panel using a defined schedule of temperature. The sample were then diluted to 2 ml by H<sub>2</sub>O, and used for analysis.

Western blotting. Immunoblotting was performed following standard procedures. The cells were incubated in a 6-well plate and grown overnight prior to treatment. Cells were exposed to 50 nM of **10** or 5 μM of triapine for different incubation times. Total cells were lysed in RIPA lysis buffer (Beyotime; Haimen, China) with a mixture of two phosphatase inhibitors and a mixture of protease inhibitors (Selleck Chemicals; Houston, TX, USA). Lysates were centrifuged at 13500 rpm/min for 20 min at 4 °C. The concentration of protein samples was determined using enhanced BCA protein assay reagent (Beyotime; Haimen, China) and total

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proteins were separated with 6% to 15% SDA-PAGE. Proteins were then transferred to PVDF membranes (Millipore; Bedford, MA, USA), blocked with 5% bovine serum albumin in Tris-buffered saline/Tween buffer for 1 h at room Primary anti-TXNIP (D5F3E, 1:1000), anti-Phospho-JNK temperature. (Thr183/Tyr185) (9251, 1:1000), anti-SAPK/JNK (9252, 1:1000), anti-p38 (8690, 1:1000), and anti-Phospho-p38 (Thr180/Tyr182) (4511, 1:1000) antibodies, all obtained from Cell Signaling Technology; Danvers, MA, USA, were incubated overnight at 4 °C, and the corresponding secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) were applied and further detected using Amersham Imager 600 system (GE Healthcare; Piscataway, NJ, USA).

**Reverse-transcription quantitative polymerase chain reaction (qPCR).** Treated and mocked-treated MV4;11 cells were harvested. Total RNA was extracted from the cells using the Trizol reagent (Invitrogen; Carlsbad, CA, USA) following the manufacturer's instructions, and the RNA concentration was quantified spectrophotometrically with the use of a Nanodrop instrument (Thermo Fisher

Scientific, Inc.; Waltham, MA, USA). Reverse-transcription PCR assay was performed in a reverse-transcription PCR system (Shanghai YEASEN Biotech Co., Ltd.; Shanghai, China) and quantified on a QuantStudio 6 Flex Real-time PCR system (Thermo Fisher Scientific, Inc.; Waltham, MA, USA) according to the manufacturer's instructions, using gene-specific primer sets and giving the results shown in Table 3. Each sample was analyzed in triplicate. The relative expression level, defined as fold change of target genes was calculated using the 2- $\Delta\Delta$ CT method. *GAPDH* and  $\beta$ -actin were used as internal controls. The expression level was normalized to the fold change detected in the corresponding control cells, which was defined as 1.0.

Table 3. Sequences of primers used for qPCR assay

| Gene    | Sequences                       |
|---------|---------------------------------|
| β-actin | F: 5'-GATGAGATTGGCATGGCTTT-3';  |
|         | R: 5'-GTCACCTTCACCGTTCCAGT-3'   |
| TXNIP   | F: 5'-CAGCCTACAGCAGGTGAGAAC-3'; |
|         | R: 5'-CTCATCTCAGAGCTCGTCCG-3'   |

F: 5'- CTTGCGGAGTGGCTAAAGTG -3'; TXNIP(2) R: 5'- TTGAAGGATGTTCCCAGAGG -3' F: 5'-CCACTCCTCCACCTTTGAC-3'; GAPDH R: 5'-ACCCTGTTGCTGTAGCCA-3'

**Measurement of mitochondrial DNA (mtDNA) copy number.** DNA samples were isolated from cells using a DNeasyblood & tissue kit (QIAGEN; Valencia, CA, USA) according to the manufacturer's instructions. Real time quantitative PCR (qPCR) was performed using SYBR Green Premix (TaKaRa; Kusatsu, Japen) on a QuantStudio 6 Flex Real-time PCR system (Thermo Fisher Scientific, Inc.; Waltham, MA, USA) with gene-specific primer sets giving the results in Table 4. Data analysis was performed with the  $\Delta\Delta$ Ct method, adjusted to the fold-change.

Table 4. Sequences of primers used for qPCR assay

| Gene | Sequence                       |
|------|--------------------------------|
| HPRT | E: 5'-TGACATGTGCCGCCTGCGAG -3' |
|      |                                |
|      |                                |
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# R: 5'-GTGGTCGCTTTCCGTGCCGA -3'

F: 5'-CACAGAAGCTGCCATCAAGTA-3';

ND2

R: 5'-CCGGAGAGTATATTGTTGAAGAG-3'

**Metabolic stability test of liver microsomes.** Buffer A was prepared with 1.0 L of 0.1 M monobasic potassium phosphate buffer containing 1.0 mM EDTA. Buffer B was prepared with 1.0 L of 0.1M dibasic potassium phosphate buffer containing 1.0 mM EDTA. Buffer C was prepared with 0.1 M potassium phosphate buffer, 1.0 mM EDTA, pH 7.4 by titrating 700 ml of buffer B with buffer A. 10 μl of a 10 mM stock solution of a reference compound (Ketanserin) was added to 190 μl MeCN to prepare 500 μM spiking solution. 1.5 μl of 500 μM spiking solution and 18.75 µl of 20 mg/ml liver microsomes were added into 479.75 µl of buffer C on ice to prepare 1.5 µM 10 spiking solution in microsomes. 6 mM NADPH stock solution was prepared by dissolving NADPH in buffer C. Then, 30 µl of 1.5 µM 10 spiking solution containing 0.75 mg/ml solution of microsomes was dispensed to the assay plates designated for different time points (0, 5, 15, 30,

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> 45 min) on ice. At 0 min, 135  $\mu$ l of MeCN containing IS was added to the wells of 0-min plate and then 15  $\mu$ l of NADPH stock solution was added. All other plates were pre-incubated at 37 °C for 5 min. Then 15  $\mu$ l of NADPH stock solution was added to the plates to start the reaction and the timing. At 5, 15, 30 and 45 min, 135  $\mu$ l of MeCN containing IS was added to the wells of the corresponding plates to stop the reaction. After quenching, the plates were shaken in a vibrator for 10 min (600 rpm/min) and then centrifuged at 5594 × *g* for 15 min. 50  $\mu$ l of the supernatant was transferred from each well into a 96-well sample plate, each well of which contained 50  $\mu$ l of ultra-pure water for LC-MS analysis.

> **Tumor xenograft experiments.** During the study, the care and use of animals was conducted in accordance with the regulations of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), laboratory animal administration in China, and the Guide for the Care and Use of Laboratory Animals. Female BALB/c nude mice were inoculated subcutaneously with 5 × 10<sup>6</sup> cells in a suspension containing 50% phenol red-free matrigel (BD Biosciences;

San Jose, CA, USA) in PBS with MV4:11 cells. In efficacy studies, tumors in mice were calipered in two dimensions twice weekly. Once the tumor size reached roughly 230 mm<sup>3</sup>, mice were randomly assigned to treatment groups. For the efficacy study, mice received either vehicle or test compound (10, 30 mg/kg, 10 ml/kg, gd) by oral gavage. Tumor volume and mouse body weight was assessed twice weekly. The major endpoint was to see if the tumor growth can be delayed or mice can be cured. Tumor size was measured twice weekly in two dimensions using a caliper, and the volume was expressed in mm<sup>3</sup> using the formula: V = $0.5a \times b^2$  where a and b are the long and short diameters of the tumor, respectively. The tumor sizes were then used for the calculations of both T-C and T/C values. T-C was calculated with T as the median time (in days) required for the treatment group tumors to reach a predetermined size (e.g., 1000 mm<sup>3</sup>), and C is the median time (in days) for the control group tumors to reach the same size. The T/C value (in percentage), an indicator of antitumor effectiveness, was calculated using the formula: T/C%=( $T_i$ - $T_0/C_i$ - $C_0$ ) ×100%, where  $T_i$  was the average tumor volume of a treatment group on a given day,  $T_0$  was the average

tumor volume of the corresponding treatment group on the day of treatment start,  $V_i$  was the average tumor volume of the vehicle control group on the same day with  $T_i$ , and  $V_0$  was the average tumor volume of the corresponding vehicle group on the day of treatment start. **Statistical analysis.** All data are presented as the mean ± standard error of mean (SEM) from at least three independent determinations, and all statistical analyses were done using the software Graphpad Prism version 6.0 (GraphPad Software, Inc.; La Jolla, CA, USA). Differences of means were tested for statistical significance with two-tailed Student's *t* test. A *P* value of < 0.05 was considered

statistically significant.

# ANCILLARY INFORMATION

# Supporting Information

The Supporting Information is available free of charge on ACS Publications website.

Molecular formula strings for compounds triapine, 1-19 (CSV)

Table of inhibition of cancer cell proliferation by **10** and triapine; figure of **10**induced cell death is not dependent on apoptosis, necroptosis or ferroptosis; UV-Vis absorption spectra of triapine or **10** and Cu<sup>2+</sup>, Fe<sup>2+</sup> and Fe<sup>3</sup>; FACS and Western blotting analysis showing triapine had no effects on TXNIP expression and mitochondrial membrane potential; <sup>1</sup>H and <sup>13</sup>C NMR spectra of compound **1**-**19** (PDF)

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# Notes

The authors declare no competing financial interest.

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# ABBREVIATIONS USED

HUVEC, human umbilical vein endothelial cell; 3-AP, traipine; EWG, electron withdrawing group; ROS, reactive oxygen species; TRX, thioredoxin; ASK1, apoptosis signaling kinase-1; JNK, c-Jun N-terminal kinase; TXNIP, thioredoxin-interacting protein; OCR, oxygen consumption rate; MAPK, mitogen-activated protein kinases; PK, pharmacokinetics; SAR, structure activity relationship; SF, selectivity factor; CI, combination index; AML, acute myeloid leukemia; NAC, N-acetyl-L-cysteine; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene;  $\alpha$ -TOC,  $\alpha$ -tocopherol; GSH, reduced glutathione; GSSG, glutathione disulfide; BCS,

bathocuproine disulfonate; TM, ammonium tetrathiomolybdate; DFO, deferoxamine; OXPHOS, oxidative phosphorylation; FITC, fluorescein isothiocyanate; PI, propidium iodide; MMP, mitochondrial membrane potential; TEM, transmission electron microscopy; ICP-MS, inductively coupled plasma mass spectrometry; mtDNA, mitochondrial DNA; nDNA, nucleus DNA.

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