Hyperthermia Selectively Destabilizes Oncogenic Fusion Proteins

Yasen Maimaitiyiming1,2,3, Qian Qian Wang1,3, Chang Yang1,3, Yasumitsu Ogra4, Yinjun Lou5, Clayton A. Smith6, Liaquat Hussain1, Yi Ming Shao7, Jiebo Lin2, Jinfeng Liu2, Lingfang Wang2, Yong Zhu8, Haiyan Lou2, Yuan Huang9, Xiaoxia Li10, Kao-Jung Chang11, Hao Chen12, Hongyan Li13, Ying Huang14, Eric Tse15, Jie Sun2, Na Bu16, Shih-Hwa Chiu17, Yan Fang Zhang5, Hao Ying Hua18, Li Ya Ma5, Ping Huang19, Ming Hua Ge19, Feng-Lin Cao10, Xiaodong Cheng16, Hongzhe Sun13, Jin Zhou10, Vasilis Vasliou8, Pengfei Xu14, Jie Jin5, Mikael Bjorklund20, Hong-Hu Zhu15, Chih-Hung Hsu2, and Hua Naranmandura1,3
ABSTRACT

The PML/RARα fusion protein is the oncogenic driver in acute promyelocytic leukemia (APL). Although most APL cases are cured by PML/RARα-targeting therapy, relapse and resistance can occur due to drug-resistant mutations. Here we report that thermal stress destabilizes the PML/RARα protein, including clinically identified drug-resistant mutants. AML1/ETO and TEL/AML1 oncofusions show similar heat shock susceptibility. Mechanistically, mild hyperthermia stimulates aggregation of PML/RARα in complex with nuclear receptor corepressors leading to ubiquitin-mediated degradation via the SIAH2 E3 ligase. Hyperthermia and arsenic therapy destabilize PML/RARα via distinct mechanisms and are synergistic in primary patient samples and in vivo, including three refractory APL cases. Collectively, our results suggest that by taking advantage of a biophysical vulnerability of PML/RARα, thermal therapy may improve prognosis in drug-resistant or otherwise refractory APL. These findings serve as a paradigm for therapeutic targeting of fusion oncprotein-associated cancers by hyperthermia.

SIGNIFICANCE: Hyperthermia destabilizes oncofusion proteins including PML/RARα and acts synergistically with standard arsenic therapy in relapsed and refractory APL. The results open up the possibility that heat shock sensitivity may be an easily targetable vulnerability of oncofusion-driven cancers.

See related commentary by Wu et al., p. 300.

INTRODUCTION

Spontaneous remissions of various cancers have been sporadically observed in patients experiencing persistent fever (1–3), especially after febrile bacterial infections (4). Consequently, inactivated mixed bacterial vaccine has been experimentally used to induce fever as a means of hyperthermia (elevated body temperatures beyond normal) in patients with cancer, leading to durable clinical remission (5–7). Although the molecular mechanisms are unclear, several studies have reported that hyperthermia has the ability to destabilize certain proteins (8–10).

There is little evidence to suggest which specific cancer types would best respond to thermal therapy. We reasoned that hyperthermia could be especially useful for targeting cancers such as acute promyelocytic leukemia (APL), which originate from specific oncogenic gene fusions (11). For example, current therapies in APL aim at elimination of the PML/RARα oncofusion protein (hereafter referred to as P/R), which is identified in >98% of APL cases (11–13). P/R protein has characteristics that could make it particularly sensitive to increased temperatures. First, P/R is relatively large (~130 kDa), and larger proteins tend to be less thermally stable (14). Second, oncogenic fusion events such as the t(15;17) chromosomal translocation in APL are unlikely to lead to a heat-stable protein in the absence of selective pressure, making these large oncofusion proteins more vulnerable to elevated temperatures.

RESULTS

Hyperthermia Destabilizes Wild-type and Drug-Resistant Mutants of P/R Fusion Protein

To evaluate the effects of hyperthermia on P/R fusion protein stability, we first used the well-characterized APL patient-derived cell line NB4. Elevating the temperature above 41°C destabilizes the PML/RARα oncofusion protein (hereafter referred to as P/R), which is identified in >98% of APL cases (11–13). P/R protein has characteristics that could make it particularly sensitive to increased temperatures.
robustly destabilized endogenous P/R in a time- and temperature-dependent manner without affecting mRNA levels (Fig. 1A; Supplementary Fig. S1A). Importantly, these mildly elevated temperatures did not significantly affect cell viability (Supplementary Fig. S1A–S1C), although they were mildly elevated temperatures did not significantly affect cell viability (Supplementary Fig. S1A–S1C), although they were mildly elevated temperatures did not significantly affect cell viability (Supplementary Fig. S1A–S1C), although they showed strong resistance to ATO or hyperthermia for 2 hours. Hsp70 protein expression was used to verify heat shock. S indicates the supernatant of RIPA lysate, and P indicates the insoluble pellet fraction. <, full-length P/R protein; , posttranslationally modified; <, degraded fragments.

Figure 1. Hyperthermia destabilizes PML/RARα oncofusion. A, Hyperthermia induced destabilization of P/R protein in NB4 cells, as assessed by Western blot using anti-PML antibody. Relative expression levels of P/R and Hsp70 mRNAs were detected by RT-qPCR. Data shown are mean ± SD (n = 3). B, Hyperthermia induced destabilization of exogenous P/R (wild-type, WT) protein in HeLa cells. C, Schematic representation of clinically reported all-trans retinoic acid (ATRA)- and arsenic trioxide (ATO)-resistant mutants in P/R fusion protein; mutations in the RARα part are numbered by codon of normal RARα.

Although rare, development of drug resistance due to mutations in P/R is a recurrent problem in APL therapy, and the prognosis in patients with recurrent drug-resistant APL, especially in those who have experienced multiple relapses, is extremely poor (15, 16). We therefore investigated the effects of hyperthermia on clinically reported all-trans retinoic acid (ATRA)- and arsenic trioxide (ATO)-resistant mutants (Fig. 1C). Hyperthermia destabilized all ATRA-resistant mutants as effectively as ATO treatment (Fig. 1D). All the ATO-resistant mutants were also destabilized by hyperthermia, although they showed strong resistance to ATO (Fig. 1E), suggesting that the mechanisms for hyperthermia- and ATO-mediated P/R destabilization are distinct. These findings exhibit that hyperthermia reduces the stability of the oncogenic P/R fusion protein, which could be potentially exploited to treat ATRA- and ATO-resistant cases.

**Hyperthermia Destabilizes P/R through Targeting the Oncogenic P/R–NCoR Complex**

Heat shock proteins play an important role in protein triage and quality control after exposure to elevated temperatures (17, 18). However, the Hsp70 and Hsp90 inhibitors VER155008 and BIIB021 had no effect on hyperthermia-induced destabilization of P/R (Supplementary Fig. S3A and S3B).
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We introduced several point mutations in LBD (numbered by mutant amino acid residues for multiple functions of P/R (20). Both CC and LBD domains played crucial roles in interacting with nuclear receptor corepressors (NCoR; Supplementary Fig. S3C and S3D), suggesting that interaction with NCoRs might be important for hyperthermia-mediated destabilization of P/R. ATRA is known to exert its therapeutic effect through dissociating NCoRs from P/R (21). Thus, we used ATRA to block the interaction of P/R with NCoRs (Supplementary Fig. S3D) to examine the involvement of NCoRs in hyperthermia-mediated P/R destabilization. ATRA completely blocked hyperthermia-induced destabilization of endogenous (Fig. 2D) and exogenous P/R (Supplementary Fig. S3E and F). Moreover, ATRA did not prevent binding of NCoR1 with the ATRA-resistant L224P mutant and was unable to inhibit the hyperthermia-mediated destabilization of this mutant (Supplementary Fig. S3G and S3H). Proximity ligation assay (PLA) and confocal experiments revealed that hyperthermia stimulated aggregation and interaction of P/R in complex with the corepressor SMRT, which was completely prevented by ATRA pretreatment (Fig. 2E; Supplementary Fig. S3I and S3J). These results suggested that the formation and aggregation of the P/R–NCoR complex played a crucial role in hyperthermia-induced destabilization of P/R.

The ligand-binding domain (LBD) harbors many important amino acid residues for multiple functions of P/R (20). We introduced several point mutations in LBD (numbered by codon of normal RARα) to impair P/R’s SUMOylation and ubiquitination (K399R; ref. 22), interaction with the NCoA1 coactivator (“LI” double mutant, L409A/I410A; ref. 23), and interaction with NCoR1/SMRT corepressors (I396E and the “AHT” triple mutant, A194G/H195G/T198A; refs. 20, 21; Fig. 2A). Among these mutants, I396E and AHT showed resistance to hyperthermia (Fig. 2F). Notably, compared with I396E, the AHT mutant displayed much weaker NCoR1 binding ability (Supplementary Fig. S4A) and had stronger resistance to hyperthermia (Fig. 2F), indicating that NCoR binding is indeed required for hyperthermia-induced destabilization of P/R. Depletion of NCoRs inhibited hyperthermia-induced destabilization of exogenous and endogenous P/R in HeLa (Fig. 2G; Supplementary Fig. S4B) as well as in NB4 cells (Supplementary Fig. S4C and S4D), respectively, which further validated the vital role of NCoRs in this context. Notably, double, but not single, knockdown of NCoR1 and SMRT inhibited hyperthermia-induced destabilization of P/R, suggesting the functional redundancy between NCoRs.

NCoRs play various roles in development and normal cellular homeostasis (24). Therefore, we directly evaluated whether hyperthermia affects the levels of NCoR1 and SMRT. This analysis showed that hyperthermia-mediated reduction of soluble NCoR1 and SMRT occurred only in the presence of wild-type (WT) P/R (Fig. 2H, top, compare lane 6 with lanes 2 and 4), but not the NCoR binding–defective P/R mutants (compare lane 6 with lanes 10 and 12). Moreover, disrupting the interaction between P/R and NCoRs by ATRA also diminished hyperthermia-mediated reduction of soluble NCoRs (Fig. 2H, lane 8). Consistently, in the endogenous P/R containing NB4 cells, NCoR1, SMRT as well as P/R were sensitive to hyperthermia, and blocking the formation of the P/R–NCoR complex by ATRA would lead to hyperthermia insensitivity of them (Fig. 2D, compare lane 4 with lane 2). Collectively, these data demonstrate that the normal PML and RARα as well as individual NCoRs are insensitive to the hyperthermia conditions used, but hyperthermia markedly destabilizes the P/R fusion protein in complex with NCoRs, which occurs only in APL cells.

Hyperthermia Leads to SIAH2-Dependent Ubiquitination of P/R

We next investigated how hyperthermia regulates P/R stability. We noticed that P/R was rapidly switched from soluble (S) form to insoluble (P) form (Supplementary Fig. S5A, top and middle), and this was accompanied by ubiquitination (Fig. 3A) without affecting the total P/R protein levels (Supplementary Fig. S5A, bottom) immediately after hyperthermia. Although ATO-mediated P/R destabilization via SUMOylation has been extensively documented (11, 25), hyperthermia induced only slight SUMOylation of P/R compared with that of ATO (Supplementary Fig. S5B, top, compare lane 3 with lane 2 and lane 6 to lane 5). In contrast, ubiquitination of the P/R protein increased in a temperature-dependent fashion (Fig. 3A).

Hyperthermia also facilitated the ubiquitination on ATRA- and ATRA-resistant P/R mutants (Supplementary Fig. S5C). In contrast, hyperthermia was unable to induce ubiquitination of AHT triple-mutant, ΔLBD mutant, or ATRA-pretreated P/R (Supplementary Fig. S5D). ATRA pretreatment neither disrupted the P/R-L224P–NCoR complex (Supplementary Fig. S5E) nor blocked hyperthermia-induced ubiquitination of this ATRA-resistant mutant (Supplementary Fig. S5E), suggesting that interaction with NCoRs is crucial for hyperthermia-mediated P/R ubiquitination. Simultaneous depletion of NCoR1 and SMRT with siRNAs largely suppressed hyperthermia-induced ubiquitination of P/R (Fig. 3B; Supplementary Fig. S5F), further supporting the above suggestion. Similarly, P/R is also critical for robust ubiquitination on NCoR1 and SMRT mediated by hyperthermia (Fig. 3C); only WT P/R but not NCoR binding–defective AHT mutant enhanced hyperthermia-mediated ubiquitination of NCoRs (Fig. 3C, compare lanes 2 and 8 with lane 4). Abolishing the interaction between P/R and NCoRs by ATRA could also repress hyperthermia- and P/R-dependent ubiquitination on NCoRs (Fig. 3C, compare lane 6 with lane 4). These data demonstrate that hyperthermia stimulates ubiquitination on the P/R–NCoR oncogenic complex but not on P/R alone or individual NCoRs.

We next investigated which E3 ligase is involved in hyperthermia-mediated ubiquitination of the P/R–NCoR complex. We reasoned that SIAH2, the known E3 ligase for NCoR1 (26), could be a likely candidate. Indeed, hyperthermia increased the interaction of SIAH2 with P/R in a PLA assay (Fig. 3D). Ubiquitination of the P/R–NCoR oncogenic complex was decreased in SIAH2-depleted cells, further supporting the involvement of this E3 ligase (Fig. 3E; Supplementary Fig. S5G). Moreover, hyperthermia enhanced the affinity of SMRT, NCoR1, and SIAH2 to WT P/R, but could not...
Figure 2. Interaction with NCoRs is essential for hyperthermia-induced P/R destabilization. A, Schematic representation of P/R fusion protein functional domains and truncation/point mutants. Mutations in the RARα part are numbered by codon of normal RARα. FL, full length; WT, wild-type. B and C, Effects of hyperthermia (HT) on WT P/R as well as its PML moiety and RARα portion functional domains truncated mutants exogenously expressed in HeLa cells. D, Inhibition of hyperthermia induced P/R, NCoR1, and SMRT destabilization in NB4 cells by ATRA pretreatment (1 μmol/L, 72 hours). E, Inhibition of hyperthermia induced P/R-SMRT interaction by ATRA pretreatment (1 μmol/L, 72 hours) in NB4 cells, as determined by the Duolink PLA assay. Quantification was performed by ImageJ software with at least 10 cells from each group and normalized to control group. Scale bar, 5 μm.

F, Effects of hyperthermia on P/R mutants (located in the RARα portion) exogenously expressed in HeLa cells. G, Inhibition of hyperthermia induced exogenous P/R (WT) destabilization by cosilencing of NCoR1 and SMRT in HeLa cells. si-NT represents nontargeting siRNA. H, Effects of hyperthermia on SMRT, NCoR1, Flag-P/R (WT) as well as its mutants. Transfected HeLa cells were pretreated with or without ATRA (2 μmol/L, 4 hours) and then subjected to heat treatment. EV stands for empty vector. Hsp70 protein expression was used to verify heat shock. S indicates the supernatant of RIPA lysate, and P indicates the insoluble pellet fraction. < expected size of the full-length P/R protein; >, posttranslationally modified; <, degraded fragments.
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Hyperthermia specifically induces ubiquitin-mediated degradation of P/R in an NCoR-dependent manner. A, Temperature-dependent ubiquitination of P/R (WT) exogenously expressed in HeLa cells, as determined by immunoprecipitation (IP) with anti-Flag antibody. B, Inhibition of hyperthermia (HT) induced exogenous P/R (WT) ubiquitination in HeLa cells by simultaneous silencing of NCoR1 and SMRT. *: IgG band. C, Effects of hyperthermia on ubiquitination of SMRT, NCoR1, and P/R. HeLa cells were cotransfected with HA-Ub and indicated expression vectors of P/R, pretreated with or without ATRA (2 \(\mu\)mol/L, 4 hours), and subjected to hyperthermia prior to IP analysis. EV, empty vector. D, Increased P/R–SIAH2 interaction by hyperthermia in HeLa cells exogenously expressing Flag-P/R, as analyzed by Duolink PLA assay. Quantification of CHX.

Effects of hyperthermia on ubiquitination of SMRT, NCoR1, and P/R. HeLa cells were cotransfected with HA-Ub and indicated expression vectors of P/R, pretreated with or without ATRA (2 \(\mu\)mol/L, 4 hours), and subjected to hyperthermia prior to IP analysis. EV, empty vector. D, Increased P/R–SIAH2 interaction by hyperthermia in HeLa cells exogenously expressing Flag-P/R, as analyzed by Duolink PLA assay. Quantification of CHX.

**Figure 3.** Hyperthermia specifically induces ubiquitin-mediated degradation of P/R in an NCoR-dependent manner. A, Temperature-dependent ubiquitination of P/R (WT) exogenously expressed in HeLa cells, as determined by immunoprecipitation (IP) with anti-Flag antibody. B, Inhibition of hyperthermia (HT) induced exogenous P/R (WT) ubiquitination in HeLa cells by simultaneous silencing of NCoR1 and SMRT. *: IgG band. C, Effects of hyperthermia on ubiquitination of SMRT, NCoR1, and P/R. HeLa cells were cotransfected with HA-Ub and indicated expression vectors of P/R, pretreated with or without ATRA (2 \(\mu\)mol/L, 4 hours), and subjected to hyperthermia prior to IP analysis. EV, empty vector. D, Increased P/R–SIAH2 interaction by hyperthermia in HeLa cells exogenously expressing Flag-P/R, as analyzed by Duolink PLA assay. Quantification was performed by ImageJ software with at least 10 cells from each group and normalized to control group. Scale bar, 5 \(\mu\)m. E, Inhibition of hyperthermia induced exogenous P/R ubiquitination by SIAH2 depletion in HeLa cells. F, Comparison of hyperthermia-induced P/R (WT) and AHT mutant’s ubiquitination as well as interactions with SMRT, NCoR1, and SIAH2 in HeLa cells. G and H, P/R degradation in the insoluble (P) fraction after hyperthermia. HeLa cells expressing Flag-P/R (WT, G) and NB4 cells (H) were subjected to hyperthermia and thereafter cultured at 37°C for 6 and 12 hours. I, Determination of the endogenous P/R protein degradation pathway by hyperthermia. NB4 cells were subjected to hyperthermia and further cultured at 37°C for 12 hours with MG132 (1 \(\mu\)mol/L) or CQ (10 \(\mu\)mol/L). J and K, Effects of proteasome and lysosome inhibitors on P/R protein degradation after hyperthermia or ATO treatment. Flag-P/R (WT) overexpressing HeLa cells were pretreated with 10 \(\mu\)g/mL cycloheximide (CHX) for 2 hours and subjected to hyperthermia or ATO (2 \(\mu\)mol/L) for 2 hours. Then, cells were further cultured at normal condition with MG132 (10 \(\mu\)mol/L) and/or CQ (20 \(\mu\)mol/L) in the presence of CHX. L, Inhibition of P/R protein degradation in p62\(^{-}\)/HeLa cells after hyperthermia. Flag-P/R (WT) was expressed in WT and p62\(^{-}\)/HeLa cells. Cells were subjected to hyperthermia and further cultured for 12 hours at normal growth condition. M, Western blot analysis of p62 expression in WT and p62\(^{-}\)/HeLa cells. Histone H3 (H3) was used as internal control of RP insoluble pellet (P) fraction. *: IgG band. N, A potential working model of the mechanism by which hyperthermia destabilizes the P/R–NCoR oncogenic complex.
stimulate the association of SIAH2 with the NCoR binding-defective AHT triple mutant; consequently, ubiquitination on AHT was nearly completely absent (Fig. 3F). Taken together, these data indicate that the E3 ligase SIAH2 plays a crucial role for hyperthermia-mediated ubiquitination on the P/R-NCoR oncogenic complex.

**Heat-Aggregated P/R Is Mainly Eliminated by the Lysosome–Autophagy Pathway**

Ubiquitination by SIAH2 could lead to removal of aggregated P/R by proteasomal degradation or the lysosome–autophagy pathway. In contrast to the rapid solubility change by hyperthermia, the kinetics of P/R protein degradation was much slower, requiring between 6 and 12 hours (Fig. 3G and H; Supplementary Fig. S5H). The ATO- and ATRA-resistant mutants, A216V and L224P, were also degraded with similar kinetics (Supplementary Fig. S5I). Both MG132, a proteasome inhibitor, and lysosome inhibitor chloroquine (CQ) reduced the degradation of hyperthermia-induced insoluble P/R (Fig. 3I, compare lanes 4 and 5 with lane 3; Fig. 3J, compare lanes 4 and 5 with lane 3; Supplementary Fig. S5H, compare lanes 7 and 8 with lane 6), whereas CQ displayed a much stronger effect, suggesting that the lysosome–autophagy pathway plays a dominant role in hyperthermia-induced P/R degradation. In comparison, the inhibitory effect of MG132 was much stronger in ATO-induced P/R degradation (Fig. 3K).

Notably, only hyperthermia- but not ATO-induced P/R degradation could be inhibited in autophagy-deficient p62−/− HeLa cells (Fig. 3L and M; Supplementary Fig. S5J), and the endogenous P/R protein showed increased ubiquitination and interaction with p62 in PLA assays (Supplementary Fig. S5K and L), further supporting the involvement of p62 in hyperthermia-mediated P/R degradation. Together, these results demonstrate that hyperthermia and ATO induce degradation of P/R through distinct pathways. Consistently, CQ also repressed hyperthermia-induced degradation of NCoR1 and SMRT (Supplementary Fig. S5L, compare lane 4 with lane 3). Moreover, the NCoR binding-defective mutants, ALBD and AHT (Supplementary Figs. S3D and S4A), could not be ubiquitinated (Fig. 3F; Supplementary Fig. S5D) or degraded (Supplementary Fig. S5N, compare lane 3 with lane 2, lane 6 with lane 5, and lane 9 with lane 8) by hyperthermia, indicating a critical role for SIAH2-dependent elimination of P/R–NCoRs by the lysosome–autophagy pathway. Figure 3N shows the proposed mechanism by which hyperthermia destabilizes P/R.

**Hyperthermia Synergistically Destabilizes P/R with ATO**

As hyperthermia and ATO destabilized P/R through different mechanisms, combination of hyperthermia with ATO might have synergistic effects. An elevated temperature (41°C) or low concentration of ATO (1 μmol/L) alone had incomplete effects on P/R destabilization in NB4 cells (Fig. 4A, compare lanes 2–5 with lane 1). Encouragingly, combination of the treatments displayed a synergistic effect (Fig. 4A, compare lanes 6 and 7 with lanes 1–5) without significantly affecting cell viability (Supplementary Fig. S6A). Synergism was also observed with exogenously expressed P/R in HeLa cells (Supplementary Fig. S6B). Although ATO alone was not able to induce ubiquitination of the A216V mutant, hyperthermia could promote ubiquitination leading to synergism (Supplementary Fig. S6C). Similarly, combination treatment more efficiently destabilized ATO- and ATRA-resistant P/R mutants (Supplementary Fig. S6D), and P/R protein in primary APL blasts from patients (Fig. 4B; Supplementary Fig. S6E) than ATO did. Even the lower-intensity hyperthermia (40°C)–ATO (0.5 μmol/L) combination treatment significantly downregulated P/R in NB4 cells (Fig. 4C). Markedly, a daily short-time (0.5 hours) low-intensity hyperthermia–ATO treatment was sufficient to dramatically reduce P/R (Fig. 4D, lanes 4, 8, and 12). Furthermore, compared with ATO alone, this mild hyperthermia–ATO treatment showed stronger effect on the induction of differentiation, derepression of the expression of P/R regulated adaptor molecule 1 (PRAM1), and reduction of cell viability in NB4 cells (Fig. 4E).

To examine the effects of hyperthermia–ATO treatment in vivo, we used fluorescently stained NB4 and patient primary APL cells transplanted in zebrafish, as this model allows the most precise control for the heat treatment in vivo. P/R protein in NB4 and primary APL cells was destabilized by hyperthermia as effectively as in vitro (Fig. 4F). Furthermore, hyperthermia–ATO combination treatment was more efficient in repressing NB4 cell proliferation in vivo than each treatment alone (Fig. 4G). These results suggest that the combination of hyperthermia with ATO is a potential therapeutic approach to treat refractory or relapsed APL for which there is no effective treatment available.

**Hyperthermia Acts Synergistically with ATO on Patients with Relapsed APL**

To preliminarily evaluate hyperthermia–ATO combination treatment in a clinical setting, we conducted case studies with one relapsing and two refractory APL patients (Fig. 5A). Patients received home-based treatment of oral arsenic (13) together with daily hyperthermia of the whole body in a temperature-controlled water bath [(42°C, 0.5 hours)/day]. Hyperthermia–ATO combination treatment showed apparent relief of tumor burden in two patients (#I and #II) with refractory central nervous system (CNS)–relapsed APL (Fig. 5A–C). The third APL patient (#III) at “Relapse 2” of the bone marrow (P/R expression: 76%) experienced the first relapse with bone marrow and extramedullary scalp involvement with a myeloid sarcoma (Fig. 5A; Supplementary Fig. S7A). The exon 5 deletion mutation in P/R was documented after the first relapse (Fig. 5A), which could not result in resistance to ATO treatment (Supplementary Fig. S7B). Then our sequencing analysis revealed the L398P mutant in the RARα portion of P/R known for causing ATRA resistance (15) and the A216V mutation in the unarranged PML but not in P/R (Supplementary Fig. S7C), which resulted in resistance to regular treatments such as ATO, ATRA, and chemotherapy (27). Our in vitro experiments in PML−/− cells showed that WT PML is sensitive to ATO, whereas PML-A216V exhibits complete resistance (Fig. 5D, compare lane 2 with lane 1 and lane 4 with lane 3). Both PML (WT) and PML-A216V associated with P/R (Supplementary Fig. S7D), but only PML-A216V impeded ATO-induced destabilization of P/R (Fig. 5D, compare lane 6 with lane 5 and lane 8 with lane 7; Fig. 5E, compare lane 2 with lane 1) as well as PML nuclear body biogenesis and SUMO1 colocalization (Supplementary
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Figure 4. Synergistic effects of hyperthermia with ATO in vitro and in a zebrafish model. A, Effects of hyperthermia or and ATO (1 μmol/L) on endogenous P/R protein levels in NB4 cells, as detected by Western blot. Quantification was performed by ImageJ software and is presented as mean ± SD (n = 3). B, Destabilization of endogenous P/R protein in primary APL cells by hyperthermia and/or ATO (1 μmol/L) for 2 hours. Data from samples of different patients were quantified by ImageJ software and presented as mean ± SD. C, Destabilization of endogenous P/R protein stability in NB4 cells by 40°C hyperthermia and/or 0.5 μmol/L ATO. Quantification was performed by ImageJ software and is presented as mean ± SD (n = 3). D, Effects of daily hyperthermia (HT) and/or ATO on P/R protein level. NB4 cells were subjected to daily hyperthermia (40°C, 0.5 h) and continuous ATO (0.5 μmol/L) treatment prior to Western blot analysis. E, Flow cytometry analysis of CD11b expression, MTT assay of cell viability, and Western blot analysis of PRAM1 expression in NB4 cells subjected to continuous ATO (0.5 μmol/L) and daily hyperthermia (40°C, 1 hour/day) for 3 days. Quantification is mean ± SD (n = 3). F, Destabilization of P/R by hyperthermia in vivo. Zebrafish carrying NB4 or primary APL cell xenografts stained with Dil as a long-term tracer were subjected to hyperthermia (41°C, 1 hour); NB4 and primary blast cells were sorted by FACS and subjected to Western blot analysis. hpf, hours post-fertilization. G, Synergistic effects of hyperthermia and ATO on NB4 cell proliferation in zebrafish. Zebrafish carrying NB4 xenografts stained with Dil as a long-term tracer were subjected to ATO (1 μmol/L) and/or hyperthermia (41°C, 0.5 h) and further cultured at 37°C for 24 hours. NB4 xenografts were visualized by confocal microscope. Cell proliferation rate was calculated by comparing the number of Dil-positive (red fluorescence) cells from each group with the control group and expressed as mean ± SD from three independent assays (n = 15 for each group).
Figure 5. Synergistic effects of hyperthermia with ATO in vivo. A, Clinical course of three patients with relapsed and drug-resistant refractory APL who received hyperthermia–ATO combination treatment (HT–ATO). Top, clinical course of the CNS-relapsed APL patient (#I), middle, clinical course of CNS-relapsed APL patient (#II) who developed a chloroma in the brain; bottom, clinical course of the patient (#III) carrying A216V mutation in the unarranged PML allele. BM, bone marrow; CR, complete remission; CSF, cerebrospinal fluid; EM, extramedullary; ICH, intracranial hemorrhage; ICP, intracranial pressure; MRD, minimal residual disease; SUV, standard uptake value. B, Wright–Giemsa staining of APL cells taken from cerebrospinal fluid of the CNS-relapsed APL allele. BM, bone marrow; CR, complete remission; CSF, cerebrospinal fluid; EM, extramedullary; ICH, intracranial hemorrhage; ICP, intracranial pressure; PML, promyelocytic leukemia. C, Positron emission tomography (PET) scanning of the head of the second relapsed APL patient (#II) before and after treatment of daily external hyperthermia [42°C, 0.5 hours]/day with ATO. PET, computed tomography (CT), merged PET/CT as well as three-dimensional MIP PET (3D MIP) images are shown. Mean and SD for the SUVs before and after hyperthermia–ATO combination treatment were calculated by analyzing four regions of interests (ROI; red circles) within the chloroma. The ROI before treatment were randomly selected, and the corresponding posttreatment positions were automatically mapped. SUVmax values were adjusted according to the background. Paired t test was used for statistical analysis. The table shows SUVmax values of the patient. D and E, Destabilization of P/R in PML−/− HeLa cells in the presence of PML–A216V and PML (WT). Cells were transfected with Flag-labeled expression vectors as indicated, pretreated with or without ATRA (2 μM/L, 4 hours), and subjected to ATO and/or hyperthermia treatment. S indicates the supernatant of RIPA lysate, and P indicates the insoluble pellet fraction. c, expected size of the full-length protein; d, degraded fragments. F, Destabilization of P/R protein in primary APL cells taken from patient #III by ATO (1 μM/L) and/or hyperthermia for 2 hours.
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Remarkably, hyperthermia induced ubiquitination (Supplementary Fig. S7F) and destabilization (Fig. 5D, compare lanes 9 and 10 with lanes 7 and 8) of PML-A216V-mediated ATO-resistant P/R and showed synergy with ATO. Furthermore, ATRA pretreatment inhibited hyperthermia-induced P/R destabilization in this context (Fig. 5D, compare lane 14 with lane 12), indicating that hyperthermia destabilized PML-A216V-mediated ATO-resistant P/R through an NCoR-dependent manner as well.

Consistent with the in vitro cell-based assays, P/R protein in primary APL cells taken from patient #III showed resistance to ATO but not hyperthermia, and hyperthermia–ATO treatment displayed strong synergistic effect for P/R destabilization (Fig. 5F). More strikingly, although 2 weeks of hyperthermia–ATO treatment did not induce appreciable changes in P/R expression (81%) in the bone marrow blasts, another 1 month of treatment resulted in a significant decrease of P/R mRNA (54%) and protein (Fig. 5A; Supplementary Fig. S7G). While this patient was receiving hyperthermia–ATO, it was found that primary blasts from the patient were sensitive to other anticancer drugs, and the patient as well as his medical team decided to shift to a compassionate drug use program. Nevertheless, during the window of hyperthermia–ATO treatment, P/R expression was significantly downregulated, which bought time for seeking alternative therapeutic options. Collectively, these results suggest hyperthermia–ATO treatment as a feasible approach to treat refractory APL. Based on the in vitro findings and the initial clinical experience with hyperthermia in relapsed/refractory APL, we have registered a clinical trial to evaluate its safety and effectiveness in a more systematic fashion (registration number ChiCTR2000035656).

**Hyperthermia Destabilizes AML1/ETO and TEL/AML1 Fusion Oncoproteins**

Because gene fusions/translocations resulting from chromosomal rearrangements belong to one of the most common mutation classes in multiple human cancers (28), we performed pilot experiments with other oncofusions including AML1/ETO of AML-M2 (29, 30) and TEL/AML1 of pre–B cell acute lymphoblastic leukemia (31). The results showed that these oncofusions are similarly aggregated and destabilized by hyperthermia (Fig. 6). Collectively, these results suggest hyperthermia as a promising approach to destabilize fusion oncoproteins, and our study primarily serves as a paradigm with clinical applications in other models.

**DISCUSSION**

Destabilizing the P/R oncogenic fusion protein is the fundamental strategy for APL therapy. Currently, ATRA- and/or ATO-based treatment is the main clinical approach to interfere with the stability and function of P/R, but relapse and resistance can occur due to drug-resistant mutations (15, 16). Here, we report that hyperthermia destabilizes both WT and drug-resistant mutant P/R but only in complex with NCoRs, leading to a selective ubiquitination-mediated degradation of these complexes via the Siah2 E3 ligase. Furthermore, combination treatment of hyperthermia and ATO showed a synergistic antileukemic effect both in vitro and in vivo. Notably, hyperthermia only marginally affected the stability of normal PML, RARα, and NCoRs. Our pilot experiments showed that hyperthermia similarly destabilizes AML1/ETO and TEL/AML1 fusion oncoproteins. Collectively, these results suggest that thermal instability of fusion oncoproteins including P/R is a biophysical vulnerability, which emerges from the oncogenic fusion event.

Fusion oncoproteins produced from chromosomal rearrangements are well-defined oncogenic drivers in multiple human cancers, making these oncofusions attractive targets for cancer treatment (28, 32). As a modality of cancer treatment, hyperthermia has long been practiced empirically by clinicians (4, 6, 7), whereas its mechanism behind the therapeutic effect is poorly understood. Thus, widespread application of hyperthermia in cancer treatment, especially in hematologic malignancies, is particularly restricted. Here, we revealed the thermal vulnerability of fusion oncoproteins as a potential mechanism of hyperthermia-based cancer treatment, opening up the possibility that heat shock sensitivity may be an easily targetable vulnerability of oncofusion-driven cancers.

It is well demonstrated that ATO elicits nuclear matrix association of P/R and subsequently promotes posttranslational modifications and degradation of the fusion protein (25, 33). We showed hyperthermia stimulating P/R–NCoR complex formation and destabilization, suggesting an ATO-independent “P/R–nuclear matrix–degradation” axis. First, hyperthermia could destabilize ATO-resistant mutants of P/R. Second, ATO but not heat treatment largely stimulated SUMOylation of P/R. Third, ATO-induced P/R degradation could mainly be inhibited by MG132, but hyperthermia-induced P/R degradation was basically suppressed by CQ. Fourth, depletion of autophagy regulator p62 abolished hyperthermia-mediated degradation but only modestly affected ATO-mediated degradation. Therefore, the mechanism for eliminating P/R by hyperthermia appears distinct from the well-characterized SUMO-triggered RNF4/ubiquitin-mediated proteasomal degradation by arsenic (11, 25).

We further found that hyperthermia leads to synergistic effects with ATO on destabilizing P/R both in vitro and in vivo. Although elevated temperatures have been reported to induce degradation of certain proteins, such as BRCA2 and HuR (8, 9), it did not directly stimulate P/R degradation. We found that hyperthermia not only enhances the interaction between P/R and NCoRs but also increases the size of P/R–NCoR-co-localized dots, suggesting hyperthermia induces abnormal aggregation of the P/R–NCoR complex. Here, only the P/R–NCoR oncogenic complex, but not the individual PML, RARα, and NCoRs, becomes insoluble, ubiquitinated, and subjected to degradation in response to hyperthermia, suggesting that hyperthermia-mediated P/R–NCoR aggregation is the trigger for subsequent destabilization of P/R. Disruption of the P/R complex with NCoRs abrogates P/R sensitivity to hyperthermia, which also supports this model (Fig. 3N).

Although additional biochemical and biophysical details of the heat-induced aggregation remain to be investigated, these may involve phase separation as heat stress associates with phase separation–controlled autophagic degradation (34).

Previously, Hugues de Thé’s group described a patient carrying rare PMLA216V (mutation in the unarranged PML allele) displaying resistance to ATO treatment (27). We consistently observed that the patient carrying rare PMLA216V (#III) showed ATO resistance, and further found that ATO barely destabilizes P/R in the blasts from this patient or PML-A216V–expressed
cell lines, which might result from PML-A216V associating with P/R and preventing ATO-mediated SUMO localization on P/R. Hyperthermia alone or in combination with ATO destabilized ATO-resistant P/R in PML-A216V–expressed cells, primary culture cells, and blasts from the patient (§III), further supporting the distinct P/R destabilization pathways by hyperthermia and ATO. In our three preliminary clinical settings, the responses of hyperthermia–ATO combination treatment are not very dramatic but interesting because disease was stabilized or slightly reduced, suggesting the clinical potential of hyperthermia. Nevertheless, our results reveal a novel mechanism for destabilizing P/R oncoproteins and suggest a prospective hyperthermia-based therapeutic strategy for refractory or relapsed APL and potentially for a wider range of cancers characterized by specific oncoprogenic fusion events (28).

**METHODS**

**Ethical Approval**

The study was approved by the Ethics Committee of the Zhejiang University School of Medicine (#2018-023) and was conducted in accordance with the Declaration of Helsinki. Written informed consents were obtained from all study participants (Supplementary Table S1; three patients) and the patients who provided samples for the study (Supplementary Table S2; six patient samples). In total, there are nine independent patients.

**Cell Lines and Primary Cultures**

Primary APL cells/blasts were freshly isolated from the bone marrow aspirates of APL patients (Supplementary Table S2) using Ficoll-Paque plus (SolarBio, 17-1440-03). The NB4 cell line was purchased from Creative Bioarray. 293T and HeLa cell lines were purchased from the Cell Bank of Chinese Academy of Sciences. Following receipt, cells were grown and frozen as a seed stock as they were available. Cell lines were authenticated using DNA fingerprinting (variable number of tandem repeats), confirmed that no cross-contamination occurred during this study, and tested for *Mycoplasma* contamination once in a year. NB4 and primary APL cells were cultured and maintained in RPMI-1640 medium (Gibco, 12800-082). All the culture media were supplemented with 10% fetal bovine serum (Gibco, 10270-106), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were kept at 37°C in 5% CO2 atmosphere. The reagents and inhibitors used to treat cells are shown in Supplementary Table S3.

**Cell Viability Assay**

**Trypan Blue Assay.** Cells were subjected to indicated treatments before the assay. HeLa cells were washed and trypsinized to obtain single-cell suspension. Cells were centrifuged, washed twice, and resuspended in PBS. Next, 0.4% trypan blue in PBS solution was mixed with the cell suspension in a 1:9 ratio (0.04% trypan blue in the mixture), incubated for 1 minute, and observed under a light microscope. Images were taken within 3 minutes, and cell viability
was calculated by comparing the number of nonstained cells with the number of all visible cells (stained plus nonstained).

**MTT Assay.** NB4 or primary APL cells were subjected to hyperthermia and/or ATO treatment, and transferred into 96-well plates. Then, 20 μL freshly made MTT solution (5 mg/mL MTT in ddH2O) was added to each well and incubated at 37°C for 4 hours. Next, 100 μL dissolving solution [10% SDS, 5% isobutanol, and 0.1% HCl in desterilized water (ddH2O)] was added into each well and further incubated at 37°C for 16 hours to completely dissolve the insoluble formazan. Cell viability and proliferation were determined by measuring the absorbance at wavelength 570 nm by a microplate reader (Thermo Multiskan FC, USA).

**Protein Expression and Gene Silencing**

Expression vectors of PML, RARα, and PML/RARα (L) were previously constructed in our lab by cloning cDNAs of respective mRNA into Flag-tagged PCMV-2B expression vector. Various mutants of P/R were constructed by homologous recombination using Con-Express II One Step Cloning Kit (Vazyme, C112-02) in line with the manufacturer’s instructions. The primer sequences for P/R mutants are listed in Supplementary Table S4. The mutants were confirmed by sequencing prior to transfection experiments. Flag-tagged expression vectors of AML1/ETO and TEL/AML1 were constructed by cloning respective coding sequences into PCMV-2B expression vector as well. For exogenous ubiquitin expression, HA-tagged PCMV expression vector was used. SIAH2 siRNAs (Hs_SIAH2_1 and Hs_SIAH2_4) and validated siRNAs targeting human NCoR1 (GS9611) and SMRT (GS9612) were purchased from Qiagen. Transfection was performed using lipofectamine 3000 (Invitrogen, L3000-008) according to the manufacturer’s instructions. Twenty-four to 36 hours after transfection, cells were treated as indicated and subjected to various analyses.

A lentiviral transduction system was used for efficient shRNA-mediated gene knockdown in NB4 cells. Lentivirus particles were produced by cotransfection of the lentiviral plasmid (pLKO.1) with helper vectors (pPAX2 and PMD2.G) into 293T cells, and viral supernatants were collected after 48 to 72 hours. Infected cells were selected with puromycin and/or hygromycin. Knockdown efficiency was assessed by Western blot. The following sequences for SMRT and NCoR1 shRNAs were used: SMRT-sh1, GCAGGCGCTACAGTGCTCAAA; SMRT-sh2, GCAGTGTAAGAACTTCATTT; NCoR1-sh1, CTATCAGCCAGAGTGGTCTAA; and NCoR1-sh2, GCCATCAACACAAATGTCAAA.

**RNA Extraction, qPCR Analysis, Sequencing Analysis, and Construction of PML−/− as well as p62−/− HeLa Cells by CRISPR-Cas9**

Total RNA was extracted using TRIzol reagent (Life Technologies) according to the manufacturer’s instructions. Total RNA (1 μg) from each group of cell sample was used as template for the reverse transcription, which was carried out using a PrimeScript RT reagent kit (Takara, RR037A). The resulting cDNAs were used as templates for qPCR or sequencing analysis. The relative expression levels of specific mRNAs were measured by real-time qPCR using the SYBR green qPCR kit (Takara, DRR820A) and analyzed on a Bio-Rad CFX-96 detection system. Gene-specific primer sequences (5’-3’) are shown in Supplementary Table S5. Region-specific primers for sequencing analysis are shown in Supplementary Table S6. Details of PML−/− HeLa cell construction by CRISPR-Cas9 technology were described in our previous article (35). The autophagy-deficient p62−/− HeLa cells were constructed by CRISPR-Cas9 technology as described (36), with GTGACGAGGAATTGACAATG as guide RNA.

**Protein Extraction and Western Blot Analysis**

Cells were washed twice with D-Hank’s solution, followed by lysis of cell pellets using RIPA lysis buffer (50 mmol/L Tris, 150 mmol/L NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.5, 0.2 mmol/L PMSF, and a complete mini protease inhibitor tablet) to obtain whole-cell lysates. Samples were incubated on ice for 15 minutes with vortexes in 5-minute intervals and centrifuged for 30 minutes at 4°C, 13,000 rpm to obtain the supernatant (S) for Western blot analysis, which is also called whole-cell lysate (NB4). The pellet (P) was washed twice with PBS, centrifuged, and lysed in LDS buffer (1 × TBS, 10% glycerol, 0.015% EDTA, 50 mmol/L DTT, and 2% SDS) by boiling for 10 minutes at 95°C. Protein concentrations were measured using the BCA Protein Quantification Kit (Yeasen Biotech, 202101ES76). Each protein sample (25 μg) was resolved by 7.5% to 12% SDS-PAGE and blotted onto PVDF membranes. The membranes were blocked with nonfat milk and incubated overnight with different primary antibodies (Supplementary Table S7) at 4°C, followed by incubation with HRP-labeled secondary antibodies (Supplementary Table S7) for 1 hour at room temperature. Then protein bands were visualized by enhanced chemiluminescence (Biological Industries; 20-500-120).

**Immunoprecipitation**

Cells were seeded in 10-cm culture dishes and transfected with 12 μg of each indicated plasmid. Twenty-four to 36 hours after transfection, cells were treated as indicated, scraped down, and collected in ice-cold PBS. Then cells were lysed by ice on sonication in IP buffer (50 mmol/L Tris-HCl pH7.5, 10% glycerol, 150 mmol/L NaCl, 2 mmol/L EDTA, 0.5% NP-40, and 1 mmol/L PMSF plus protease inhibitors). Immunoprecipitation experiments were performed using protein A/G PLUS-Agarose Immunoprecipitation Reagent (CST, sc-2003) according to the manufacturer’s instructions.

**Immunofluorescence Microscopy**

NB4 or primary APL cells were transferred onto glass slides using a Shandon cytospin (Runcorn, UK). HeLa cells were grown in culture plates or wells containing glass disks and transferred onto glass slides following indicated treatments. Slides were washed twice with PBS, fixed in 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100. Slides were blocked with 2% BSA in PBS, followed by incubation with primary antibodies (Supplementary Table S7) overnight at 4°C. The next day, slides were washed thrice with PBS and incubated with fluorescent-labeled secondary antibodies (Supplementary Table S7) at room temperature for 4 hours and washed thrice with PBS. Slides were mounted using DAPI Fluoromount-G (SouthernBiotech, 0100-20) and stored in the dark at 4°C. The fluorescent signals were visualized under a Zeiss (Göttingen, Germany) 510 confocal microscope.

**Duolink PLA**

Duolink In Situ Red kit (Sigma, DUO92101) was used for PLA according to the manufacturer’s instructions. NB4 or primary APL cells were transferred onto glass slides using a Shandon cytospin (Runcorn). Adherent cells were seeded in wells or culture plates containing glass disks, subjected to indicated treatments, and transferred onto glass slides. The slides were fixed by 4% PFA, washed three times with PBS, permeabilized with 0.1% Triton X-100, and blocked by Duolink blocking buffer. Slides were incubated with primary antibodies (Supplementary Table S7) at 4°C overnight with gentle agitation. PLA assay was performed in line with the manufacturer’s instructions.

**Wright–Giemsa Staining**

Primary APL cells taken from cerebrospinal fluid of patients who had undergone hyperthermia–ATO combination treatment were loaded onto glass slides, fixed in methanol for 5 to 7 minutes, and air dried. Giemsa Stain (Sigma, GS900) was diluted 1:20 with ddH2O, and the slides were stained for 15 minutes. Then slides were rinsed in ddH2O three times, air dried, and observed in the bright field of a high-resolution microscope.
Histologic and IHC Staining

The dissected lesion (myeloid sarcoma) of the extramedullary scalp relapsed APL patient (###) was subjected to hematoxylin–eosin (H&E) and IHC staining in line with standard protocols. Briefly, dewaxed and endogenous peroxidase activity blocked sections were either stained with H&E or incubated with primary antibodies for BCL-2, MPO, and Ki67, followed by incubation with secondary antibodies. All of staining results were double-blind judged and interpreted by different pathologists.

Flow Cytometry Analysis of CD11b Expression

ATO- and/or hyperthermia-treated NB4 cells were centrifuged, washed with PBS, and counted. Then 2 × 10^6 cells were taken and incubated with FITC-conjugated anti-CD11b antibody at 37°C for 30 minutes with gentle agitation. Cells were washed twice with PBS and resuspended. Subsequently, expression of CD11b in each group was measured by a flow cytometer.

NB4 and Primary APL Cell Staining by DiI and Transplantation into Zebrafish

DiI Staining of NB4 and Primary Cells. NB4 or primary APL cells were seeded in culture dishes and grown to 80% confluency, counted, centrifuged (200 × g for 4 minutes), and resuspended in PBS. Then 3 × 10^6 cells were collected in a 15-mL centrifuge tube, washed with PBS, and centrifuged (200 × g for 4 minutes). Meanwhile, 10 mmol/L stock solution of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) was diluted into 2 μmol/L working solution in prewarmed PBS. The supernatant was discarded, and cells were then incubated in 37°C water bath in 3 mL cold PBS containing 2 μmol/L DiI for 5 minutes, followed by 15-minute incubation at 4°C with gentle agitation. The cells were centrifuged and washed twice with PBS, resuspended in 1 mL cold PBS, and placed on ice.

Transplantation of DiI-Stained NB4 and Primary APL Cells into Zebrafish. DiI-stained NB4 and primary APL cells were injected into the pericardium of 30 hpf (hours postfertilization) zebrafish embryos at a stage prior to the development of adaptive immune system using 30 ½ gauge needle (BD, 50–100 cells/fish). Recipient larvae were raised at 37°C in sterilized fish culture water for 24 hours. NB4 and primary APL cell–transplanted fish were observed by fluorescence microscope, and only the ones with stained cells evenly distributed throughout the circulation were selected for subsequent experiments. These animals were randomly distributed into four groups and subjected to indicated treatments. After 24 hours, the animals from each group were observed under fluorescence microscope to visualize DiI-stained cells. Cell proliferation rate was calculated by counting the number of NB4 cells from each group and comparing with that of control.

FACS by Flow Cytometry

DiI-stained NB4 cells were injected into the pericardium of 30 hpf embryos, cultured for 24 hours, and subjected to indicated treatment. Larvae were amputated, crushed, and trypsinized. Cells were centrifuged and washed with PBS containing 5% FBS, filtered through a 40-μm cell strainer, and counted. Sorting was conducted on a Beckman moFlo Astrios EQ at excitation wavelength of 561 nm and collected in PBS containing 5% FBS on ice. The cells were subjected to Western blot analysis to determine changes of P/R protein.

Hyperthermia andArsenic Treatment of Cells, NB4-Transplanted Zebrafish, and APL Patients

Culture flasks or dishes (containing cells or zebrafish) were tightly sealed and completely immersed in a thermostar bath at the indicated temperature for the indicated time. Hyperthermia treatment of patients (Supplementary Table S1) was started after obtaining institutional review board approval (#2018-023) and taking written informed consents from patients. Preheated thermostat bathtub was used for hyperthermia treatment. After administration of oral arsenic (Realgar-Indigo Naturalis Formula, RIF, 60 mg/kg), patients were assigned to take the hot bath (–42°C) for 30 minutes per day under the supervision of adult family members with close monitoring of platelet and white blood cell counts. Heat treatment was immediately stopped if the patient developed symptoms including headache, vomiting, or fatigue.

Statistical Analysis

Each experiment was performed at least three times. Statistical analysis was carried out using one-way ANOVA or paired t test followed by proper posttest (Signamplot, Systat Software Inc), and a probability value of less than 0.05 (*, *P < 0.05) was accepted as a significant difference; **, P < 0.01; #, P < 0.001.

Data and Materials Availability

All data are available in the main text or the supplementary materials.

Authors’ Disclosures

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Authors’ Contributions

Y. Maimaitiyiming: Conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, writing—original draft, project administration, writing—review and editing.
Q.Q. Wang: Data curation, formal analysis, funding acquisition, validation, investigation, visualization, writing—original draft, writing—review and editing.
Y. Lou: Resources, data curation, and methodology.
C.A. Smith: Writing—review and editing. L. Hussain: Data curation, investigation. J. Li: Data curation, investigation, writing—review and editing. Y. Yang: Data curation, formal analysis, funding acquisition, validation, investigation, visualization, writing—original draft, writing—review and editing. Y. Ogra: Resources, methodology, writing—review and editing. Y. L.: Resources, data curation, and methodology.
C. Yang: Data curation, formal analysis, funding acquisition, validation, investigation, visualization, methodology, writing—review and editing.
Y. Ogra: Resources, methodology, writing—review and editing.
Y. Huang: Resources, data curation, methodology.
X. Li: Resources, data curation, methodology.
Y. Huang: Resources, data curation, methodology.
H. Li: Resources, methodology.
J. Liu: Data curation, investigation, writing—review and editing.
C. Wang: Data curation, writing—review and editing.
Y. Zhu: Methodology, writing—review and editing.
Y. Lou: Resources, data curation, methodology.
H. Huang: Resources, data curation, methodology.
Y. Huang: Resources, data curation, methodology.
H. Li: Resources, methodology.
Y. Huang: Resources, data curation, methodology.
H. Liu: Resources, methodology.
J. Liu: Data curation, investigation, writing—review and editing.
C. Wang: Data curation, writing—review and editing.
Y. Zhang: Resources, data curation, methodology.
H. Ma: Resources, data curation.
S. Bu: Resources, data curation.
C. Yang: Formal analysis, methodology.
M. Ge: Resources, methodology.
Y. Huang: Resources, methodology.
J. Jin: Resources, methodology.
H. Hsu: Conceptualization, formal analysis, supervision, funding acquisition, validation, investigation, visualization, methodology, writing—original draft, project administration, writing—review and editing.
H. Hsu: Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, validation, methodology, writing—original draft, project administration, writing—review and editing.
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Yasen Maimaitiyiming, Qian Qian Wang, Chang Yang, et al.


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