Hyperthermia Selectively Destabilizes Fusion Oncoproteins

Yasen Maimaitiyiming, Qian Qian Wang, Chang Yang, Yasumitsu Ogra, Yinjun Lou, Clayton A. Smith, Liaquat Hussain, Yi Ming Shao, Jiebo Lin, Jinfeng Liu, Lingfang Wang, Yong Zhu, Haiyan Lou, Yuan Huang, Xiaoxia Li, Kao-Jung Chang, Hao Chen, Hongyan Li, Ying Huang, Eric Tse, Jie Sun, Na Bu, Shih-Hwa Chiu, Yan Fang Zhang, Hao Ying Hua, Li Ya Ma, Ping Huang, Ming Hua Ge, Pengfei Xu, Jie Jin, Mikael Bjorklund, Hong-Hu Zhu, Chih-Hung Hsu, Hua Naranmandura

1Department of Hematology of First Affiliated Hospital, and Department of Public Health, Zhejiang University School of Medicine, Hangzhou, China; 2Women's Hospital, and Institute of Genetics, and Department of Environmental Medicine, Zhejiang University School of Medicine, Hangzhou, China; 3Zhejiang Laboratory for Systems & Precision Medicine, Zhejiang University Medical Center, Hangzhou, China. 4Department of Toxicology, Graduate School of Pharmaceutical Sciences, Chiba University, 1-8-1 Inohana, Chuo, Chiba, Japan; 5Department of Hematology, First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China; 6Blood Disorders and Cellular Therapies Center, University of Colorado Hospital, Denver, USA; 7Department of Pharmacology, Inner Mongolia Medical University, Hohhot 010000, China. 8Department of Environmental Sciences, Yale University School of Public Health, Hew Haven, Connecticut, USA; 9Zhejiang Province Lishui Municipal Hospital; 10Department of Hematology, the First Affiliated Hospital, Harbin Medical University, Harbin, China; 11Institute of Clinical Medicine, National Yang Ming University, Taipei, Taiwan, ROC; 12Division of Newborn Medicine and Program in Epigenetics, Children's Hospital, Harvard Medical School, 300 Longwood Avenue, Boston, MA, 02115, USA; 13Department of Chemistry, the University of Hong Kong, Pokfulam, Road, Hong Kong, P.R. China; 14Institute of Genetics, Zhejiang University, and Department of Genetics, School of Medicine, Zhejiang University, Hangzhou, China; 15Department of Medicine, the University of Hong Kong and Queen Mary Hospital, Hong Kong, P.R. China; 16Women's Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang, China; 17Taipei Veterans General Hospital Department of Medical Research, Taipei, Taiwan, ROC; 18Southern Medical University, Guangzhou, China; 19Zhejiang Provincial People's Hospital, Hangzhou, China; 20Zhejiang University-University of Edinburgh Institute, Zhejiang University School of Medicine, Hangzhou, China.

#These authors contributed equally to this work.

Chih-Hung Hsu and Hua Naranmandura jointly supervised this work.
* Correspondence to:
Dr. Chih-Hung Hsu, Women's Hospital, and Institute of Genetics, and Department of Environmental Medicine, Zhejiang University School of Medicine, Hangzhou, Zhejiang, 310058, China, E-mail: ch_hsu@zju.edu.cn;

Dr. Hua Naranmandura, Department of Public Health, and Department of Hematology of First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang, 310058, China, E-mail: narenman@zju.edu.cn.

Running Title: Destabilizing the PML/RARα Oncofusion by Hyperthermia.

Keywords: Hyperthermia; Acute promyelocytic leukemia; Fusion oncoprotein; Drug resistance; Nuclear receptor corepressors.

Conflict of interest statement
The authors declare no potential conflicts of interest.

Abstract: PML/RARα fusion protein is the oncogenic driver in acute promyelocytic leukemia (APL). While 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 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 SIAH2 E3 ligase. Hyperthermia and arsenic therapy destabilize P/R via distinct mechanisms and are synergistic in primary patient samples and in vivo including 3 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 oncoprotein associated cancers by hyperthermia.

Statement of Significance: Hyperthermia destabilizes oncofusion proteins including PML/RARα and acts synergistically with standard arsenic therapy in relapsed and refractory acute promyelocytic leukemia. The results open up the possibility that heat shock sensitivity may be an easily targetable vulnerability of oncofusion-driven cancers.
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 cancer patients leading to durable clinical remission (5-7). While 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 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 as P/R), which is identified in >98% of APL cases (11-13). P/R protein has characteristics, which 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 (HT) on P/R fusion protein stability, we first used the well-characterized APL patient derived cell line NB4. Elevating the temperature above 41°C 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-C), although were sufficient to destabilize P/R fusion protein (Fig. 1A; Supplementary Fig. S1A) and derepress P/R-target genes (Supplementary Fig. S1D). The stability of exogenous P/R protein in HeLa cells was similarly affected by hyperthermia (Fig. 1B; Supplementary Fig. S1E-G). As a control, hyperthermia increased Hsp70 mRNA or protein expression (Fig. 1A, B; Supplementary Fig. S1A, E, F). Analysis of unfused normal RARα and PML showed that these proteins are almost insensitive to hyperthermia (Supplementary Fig. S2). Therefore, the fusion between two normally stable proteins leads to a thermolabile P/R oncofusion.

Although rare, development of drug resistance due to mutations in P/R is a recurrent problem in APL therapy, and the prognosis in recurrent drug resistant APL patients, especially in those 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 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-NCoRs complex

Heat-shock proteins play an important role in protein triage and quality control after exposure to
elevated temperatures (17, 18). However, Hsp70 and Hsp90 inhibitors VER155008 and BIIB021
had no effect on hyperthermia-induced destabilization of P/R (Supplementary Fig. S3A, B),
implying that these heat-shock proteins are not the key participants in this process. We next
constructed a series of truncation mutants to identify the domains contributing to P/R
destabilization by hyperthermia (Fig. 2A). Among these, deletion of the coiled-coil domain
(ΔCC) led to partial resistance (Fig. 2B) and deletion of the ligand-binding domain (ΔLBD)
resulted in almost complete resistance to hyperthermia (Fig. 2C). Consistent with previous
studies (19, 20), both CC and LBD domains played crucial roles in interacting with nuclear
receptor corepressors (NCoRs) (Supplementary Fig. S3C, D), 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, 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, H). Proximity ligation assay (PLA) and
confocal experiments revealed that hyperthermia stimulated aggregation and interaction of P/R
in complex with corepressor SMRT, which was completely prevented by ATRA pretreatment
(Fig. 2E, Supplementary Fig. S3I, J). These results suggested that the formation and aggregation
of P/R-NCoRs complex acted a crucial role for 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) (22), interaction with
NCoA1 coactivator (“LI” double mutant, L409A/I410A) (23) and interaction with
NCoR1/SMRT corepressors (I396E and the “AHT” triple mutant, A194G/H195G/T198A) (21)
(Fig. 2A). Among these mutants, I396E and AHT showed resistance to hyperthermia (Fig. 2F).
Notably, compared to I396E, AHT mutant displayed much weaker NCoR1 binding ability (Supplementary Fig. S4A) and had stronger resistance to hyperthermia (Fig. 2F), indicating NCOrs-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, D) 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.

Nuclear receptor corepressors 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 only occurred in the presence of wild-type P/R (Fig. 2H, top panel, compare lane 6 to lanes 2 and 4), but not the NCOrs-binding defective P/R mutants (compare lane 6 to 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 P/R-NCOrs complex by ATRA would lead to hyperthermia insensitivity of them (Fig. 2D, compare lane 4 to 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 P/R fusion protein in complex with NCOrs, which only occurs 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 panel) and this was accompanied by ubiquitination (Fig. 3A) without affecting the total P/R protein levels (Supplementary Fig. S5A bottom panel) 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 to that of ATO (Supplementary Fig. S5B, top panel, compare lane 3 to 2 and 6 to 5). In contrast, ubiquitination of P/R protein increased in a temperature-dependent fashion (Fig. 3A).

Hyperthermia also facilitated the ubiquitination on ATRA- and ATO-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)-NCOrs complex (Supplementary Fig. S3G) nor blocked hyperthermia-induced ubiquitination of this ATRA-resistant mutant (Supplementary Fig. S5E), suggesting that interacting with NCOrs are 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 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 NCoRs-binding defective AHT mutant enhanced hyperthermia-mediated ubiquitination of NCoRs (Fig. 3C, compare lanes 2, 8 to 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 to 4). These data demonstrate that hyperthermia stimulates ubiquitination on the P/R-NCoRs oncogenic complex but not on P/R alone or individual NCoRs.

We next investigated which E3 ligase is involved in hyperthermia-mediated ubiquitination of P/R-NCoRs 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 P/R-NCoRs 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 stimulate the association of SIAH2 to the NCoRs-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 P/R-NCoRs oncogenic complex.

**Heat-aggregated P/R is mainly eliminated by lysosome-autophagy pathway**

Ubiquitination by SIAH2 could lead to removal of aggregated P/R by proteasomal degradation or 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 to 12 hours (Fig. 3G, 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, 5 to lane 3; Fig. 3J, compare lanes 4, 5 to lane 3; Supplementary Fig. S5H, compare lanes 7, 8 to lane 6), while CQ displayed a much stronger effect suggesting that 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, M; Supplementary Fig. S5J), and the endogenous P/R protein showed increased ubiquitination and interaction with p62 in PLA assays (Supplementary Fig. S5K, L), further supporting the involvement of p62 in hyperthermia-mediated P/R degradation. Together, these results demonstrated 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. S5M, compare lane 4 to 3). Moreover, the NCoRs-binding defective mutants, ΔLBD and AHT (Supplementary Fig. S3D, S4A), could not be ubiquitinated (Fig. 3F; Supplementary Fig. S5D) or degraded (Supplementary Fig. S5N, compare lanes 3 to 2, 6 to 5, 9 to 8) by hyperthermia, indicating a critical role for SIAH2-dependent elimination of P/R-NCoRs by 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 μM) alone had incomplete effects on P/R destabilization in NB4 cells (Fig. 4A, compare lanes 2-5 to 1). Encouragingly, combination of the treatments displayed a synergistic effect (Fig. 4A, compare lanes 6, 7 to 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 lower-intensity hyperthermia (40°C) - ATO (0.5 μM) combination treatment significantly down-regulated P/R in NB4 cells (Fig. 4C). Markedly, a daily short-time (0.5 h) low-intensity hyperthermia-ATO treatment was sufficient to dramatically reduce P/R (Fig. 4D, lanes 4, 8, and 12). Furthermore, compared to ATO alone, this mild hyperthermia-ATO treatment showed stronger effect on induction of differentiation, de-repression 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 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 relapsed APL patients**

To preliminarily evaluate hyperthermia-ATO combination treatment in clinical setting, we conducted case studies with one relapsing and two refractory APL patients (Fig. 5A). Patients
received home-based treatment of oral arsenic (27) together with daily hyperthermia of the whole body in a temperature-controlled water bath [(42°C, 0.5 h)/day]. Hyperthermia-ATO combination treatment showed apparent relief of tumor burden on two patients (#I, #II) with refractory 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 L398P mutant in the RARα portion of P/R known for causing ATRA resistance (15), and 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 (28).

Our in vitro experiments in PML−/− cells showed that wild type PML is sensitive to ATO, while PML-A216V exhibits complete resistance (Fig. 5D, compare lanes 2 to 1 and 4 to 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 lanes 6 to 5 and 8 to 7; Fig. 5E, compare lane 2 to 1) as well as PML nuclear bodies’ biogenesis and SUMO1 colocalization (Supplementary Fig. S7E, compare panel 4 with panel 2). Remarkably, hyperthermia induced ubiquitination (Supplementary Fig. S7F) and destabilization (Fig. 5D, compare lanes 9-10 to 7-8) 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 to 12), indicating that hyperthermia destabilized PML-A216V-mediated ATO-resistant P/R through a NCoRs dependent manner as well.

Consistent with the in vitro cell-based assays, P/R protein in primary APL cells taken from the 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 two weeks of hyperthermia-ATO treatment did not induce appreciable changes in P/R expression (81%) in the bone marrow blasts, another one-month treatment resulted in a significant decrease of P/R mRNA (54%) and protein (Fig. 5A; Supplementary Fig. S7G). While receiving hyperthermia-ATO, it was found that primary blasts from this 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 down-regulated, which bought time for seeking alternative therapeutic options. Collectively, these results suggest the 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 No. ChiCTR2000035656).
Hyperthermia destabilizes AML1/ETO and TEL/AML1 fusion oncoproteins

Since gene fusions/translocations resulting from chromosomal rearrangements belong to one of the most common mutation classes in multiple human cancers (29), we performed pilot experiments with other oncofusions including AML1/ETO of AML-M2 (30, 31), and TEL/AML1 of pre-B cell acute lymphoblastic leukemia (32). 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 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 wild type and drug-resistant mutant P/R, but only in complex with NCoRs, leading to a selective ubiquitination-mediated degradation of these complexes via SIAH2 E3 ligase. Furthermore, combination treatment of hyperthermia and ATO showed a synergistic anti-leukemic 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 (29, 33). As a modality of cancer treatment, hyperthermia has long been practiced empirically by clinicians (4, 6, 7), while its mechanism behind the therapeutic effect is poorly understood. Thus, widespread application of hyperthermia in cancer treatment, especially in hematological 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 post-translational modifications and degradation of the fusion protein (25, 34). We showed hyperthermia stimulating P/R-NCoRs 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 BRAC2 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-colocalized dots, suggesting hyperthermia induces abnormal aggregation of P/R-NCoRs complex. Here, only P/R-NCoRs oncogenic complex but not the individual PML, RARα, and NCoRs become insoluble, ubiquitinated, and subjected to degradation in response to hyperthermia, suggesting that hyperthermia-mediated P/R-NCoRs aggregation is the trigger of subsequent destabilization of P/R. Disruption of P/R complex with NCoRs abrogates P/R sensitivity to hyperthermia, which also supports this model (Fig. 3N). While 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 (35).

Previously, Hugues de Thé’s group described a rare PML-A216V-carried patient (mutation in the unarranged PML allele) displaying resistance to ATO treatment (28). We consistently observed that the PML-A216V-carried patient (#III) showed ATO resistance, and further found that ATO barely destabilize 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 clinical potentials of hyperthermia. Nevertheless, our results reveal a novel mechanism for destabilizing P/R oncofusion and suggest a prospective hyperthermia-based therapeutic strategy for refractory or relapsed APL and potentially for a wider range of cancers characterized by specific oncogenic fusion events (29).

**Methods**

**Ethical Approval**
The study was approved by the Ethics Committee of 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 (Table S1, 3 patients) and the patients who provided samples for the study (Table S2, 6 patient samples). In total, there are 9 independent patients.

**Cell Lines and Primary Cultures**

Primary APL cells/blasts were freshly isolated from the bone marrow aspirates of APL patients (Table S2) using Ficoll-Paque plus (SolarBio, 17-1440-03). NB4 cell line was purchased from Creative Bioarray. 293T and HeLa cell lines were purchased from 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 all cell lines were tested for mycoplasma contamination once in a year. NB4 and primary APL cells were cultured and maintained in RPMI-1640 medium (Gibco, 12800-017), 293T and HeLa cells were cultured and maintained in DMEM (Gibco, 12800-082) medium. All the culture medium 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 were shown in 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 1:9 ration (0.04% trypan blue in the mixture), incubated for 1 min, and observed under a light microscope. Images were taken within 3 min and cell viability was calculated by comparing the number of non-stained cells to the number of all visible cells (stained plus non-stained).

**MTT assay**

NB4 or primary APL cells were subjected to HT 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 h. 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 h to completely dissolve the insoluble formazan. Cell viability and proliferation was 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 ClonExpress II One Step Cloning Kit (Vanzyme, C112-02) in line with 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 (CDS) 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. 24–36 h after transfection, cells were treated as indicated and subjected to various analysis.

Lentiviral transduction system was used for efficient shRNA mediated gene knockdown in NB4 cells. Lentivirus particles were produced by co-transfection of the lentiviral plasmid (pLKO.1) with helper vectors (psPAX2 and PMD2.G) into 293T cells, and viral supernatants were collected after 48-72 h. Infected cells were selected with puromycin and/or hygromycin. Knockdown efficiency was assessed by western blot. The sequences for SMRT and NCoR1 shRNAs are listed below: SMRT-sh1, GCAGCGCATCAAGTTCATCAA; SMRT sh2, GCAGTGTAAGAACTTCTACTT; NCoR1-sh1, CTATCAGCCAGAGGTTGTTAA; NCoR1-sh2, GCCATCAAACACAAATGTCAAA.

**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. 1 μg of total RNA 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 quantitative PCR (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') were shown in Supplementary Table S5. Region specific primers used for sequencing analysis were shown in Table S6. Details of PML−/− HeLa cells’s construction by CRISPR-Cas9 technology were described in our previous article (36). The autophagy deficient p62−/− HeLa cells were constructed by CRISPR-Cas9 technology as described (37), with GTGACGAGGAATTGACAATG as guide RNA.

**Protein Extraction and Western Blot Analysis**

12
Cells were washed twice with D-Hank’s solution, followed by lysis of cell pellets using RIPA lysis buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.5, 0.2 mM PMSF, and a complete mini protease inhibitor tablet) to obtain whole cell lysates. Samples were incubated on ice for 15 min with vortexes in 5 min interval and centrifuged for 30 min at 4°C, 13000 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 mM DTT, and 2% SDS) by boiling for 10 min at 95°C. Protein concentrations were measured using BCA Protein Quantification Kit (Yeasen Biotech, 20201ES76). 25 μg of each protein sample was resolved by 7.5~12% SDS-PAGE and blotted onto PVDF membranes. The membranes were blocked with non-fat milk and incubated overnight with different primary antibodies (Table S7) at 4°C, followed by incubation with HRP-labeled secondary antibodies (Table S7) for 1 h 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. 24~36 h after transfection, cells were treated as indicated, scraped down and collected in ice-cold PBS. Then cells were lysed on ice by sonication in IP buffer (50mM Tris-HCl pH7.5, 10% glycerol, 150 mM NaCl, 2 mM EDTA, 0.5% NP-40, 1 mM 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 (Table S7) overnight at 4°C. Next day, slides were washed thrice with PBS and incubated with fluorescent-labeled secondary antibodies (Table S7) at room temperature for 4 h and washed thrice with PBS. Slides were mounted using DAPI Fluoromount-G® (SouthernBiotech, 0100-20) and stored in dark at 4°C. The fluorescent signals were visualized under a Zeiss (Göttingen, Germany) 510 confocal microscope.

**Duolink Proximity Ligation Assay (PLA)**

Duolink® In Situ Red kit (Sigma, DUO92101) was used for PLA according to manufacturer’s instructions. NB4 or primary APL cells were transferred onto glass slides using a Shandon cytospin (Runcorn, UK). 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 3 times with PBS, permeabilized with 0.1% Triton X-100, and blocked by Duolink blocking buffer. Slides were incubated with primary antibodies (Table S7) at 4°C overnight with gentle agitation. PLA assay was performed in line with the manufactures’ instructions.

**Wright-Giemsa Staining**

Primary APL cells taken from cerebrospinal fluid of patients who undergone HT-ATO combination treatment were loaded onto glass slides, fixed in methanol for 5-7 minutes and air dried. Giemsa Stain (Sigma, GS500) was diluted 1:20 with ddH$_2$O and the slides were stained for 15 min. Then slides were rinsed in ddH$_2$O for 3 times, air dried and observed in the bright field of a high-resolution microscope.

**Histological and Immunohistochemical Staining**

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

**Flow Cytometry Analysis of CD11b Expression**

ATO or/and hyperthermia treated NB4 cells were centrifuged, washed with PBS and counted. 2×10$^5$ cells were taken and incubated with FITC-conjugated anti-CD11b antibody at 37°C for 30 min with gentle agitation. Then, 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% confluence, counted, centrifuged (200 g, 4 min) and resuspended in PBS. 3×10$^6$ cells were collected in 15 mL centrifuge tube, washed with PBS and centrifuged (200 g, 4 min). Meanwhile, 10 mM stock solution of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) was diluted into 2 μM working solution in pre-warmed PBS. The supernatant was discarded and cells were then incubated at 37°C waterbath in 3 mL PBS containing 2 μM DiI for 5 min, followed by 15 min incubation at 4°C with gentle agitaion. 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 post-fertilization) 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 h. NB4 and primary APL cell transplanted fishes 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 4 groups and subjected to indicated treatments. After 24 h, the animals from each group were observed under fluorescence microscope to visualize Dil stained cells. Cell proliferation rate was calculated by counting the number of NB4 cells from each group and comparing with that of control.

Fluorescence Activated Cell sorting (FACS) by Flow Cytometry
Dil-stained NB4 cells were injected into pericardium of 30 hpf (hours post-fertilization) embryos, cultured for 24 h 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 and Arsenic 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 thermostat water-bath at indicated temperature for indicated time. Hyperthermia treatment of patients (Table S1) were 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 min per day at 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 etc.

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 post-test (Sigmaplot, Systat Software Inc), and a probability value of less than 0.05 (*p<0.05) was accepted as a significant difference, ** represents p<0.01, # represents p<0.001.

Data and materials availability
All data is available in the main text or the supplementary materials.

Author 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; C. Yang: Data curation, formal analysis, funding acquisition, validation, investigation, visualization, methodology, writing-review and editing; Y. Ogra: Methodology, resources, writing-review and editing; Y. Lou: Data curation, resources, methodology; C. Smith: Writing-review and editing; L. Hussain: Investigation, data curation; Y. M. Shao: Investigation, data curation; J. Lin: Investigation, data curation, writing-review and editing; J. Liu: Investigation, data curation, writing-review and editing; L. Fang: Data curation, writing-review and editing; Y. Zhu: Methodology, writing-review and editing; H. Y. Lou: Methodology, resources, data curation; Y. Huang: Methodology, resources, data curation; X. Li: Methodology, resources, data curation; K. J. Chang: Methodology, formal analysis; H. Chen: Methodology, formal analysis; H. Li: Methodology, resources; Y. Huang: Methodology, resources, data curation; E. Tse: Methodology, resources; J. Sun: Resources, data curation; N. Bu: Resources, data curation; S. H. Chiou: Methodology, formal analysis; Y. F. Zhang: Resources, data curation; H. Y. Hua: Methodology, resources; L. Y. Ma: Resources, data curation; P. Huang: Methodology, resources; M. H. Ge: Methodology, resources; F. L. Cao: Methodology, resources; X. Cheng: Methodology, writing-review and editing; H. Sun: Methodology, writing-review and editing; J. Zhou: Resources, writing-review and editing; V. Vasiliou: Resources, writing-review and editing; P. Xu: Methodology, resources; J. Jin: Resources, writing-review and editing; M. Bjorklund: Formal analysis, writing-review and editing; H.H. Zhu: Resources, investigation, validation, writing-review and editing; C.H. Hsu: Conceptualization, formal analysis, supervision, funding acquisition, visualization, validation, investigation, methodology, writing-original draft, project administration, writing-review and editing; H. Naranmandura: Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, validation, methodology, writing-original draft, project administration, writing-review and editing. All authors read and approved the final draft of the manuscript.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (No. 81872942, 31972883, 82000155, 82003875), the China Postdoctoral Science Foundation (No. 2020M671765, 2020M681900, 2020M681901), and the Zhejiang Provincial Program for the Cultivation of High-Level Innovative Health Talents. We thank the Core Facilities of Zhejiang University School of Medicine for the technical support. We thank Abclonal for supplying some of the antibodies in the study. We thank Chain Medical Laboratory Co., Ltd China for the support of patient mutation sequencing.
References:


Figure Legends and Figures

Fig. 1. Hyperthermia destabilizes PML/RARα oncofusion. A, Hyperthermia (HT) 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 is mean ± (standard deviation, SD), n=3. B, Hyperthermia induced destabilization of exogenous P/R (WT) protein in HeLa cells. C, Schematic representation of clinically reported ATRA- and ATO-resistant mutants in P/R fusion protein, mutations in RARα part are numbered by codon of normal RARα. D and E, Hyperthermia induced destabilization of ATRA- and ATO- resistant P/R mutants. Transfected HeLa cells were subjected to 2 μM ATO or hyperthermia for 2 h. Hsp70 protein expression was used to verify heat-shock. S indicates the supernatant of RIPA lysate and P indicates the insoluble pellet fraction. (<>) indicates the full-length P/R protein, (▶) post-translationally modified, and (▶) degraded fragments.
Fig. 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 RARα part are numbered by codon of normal RARα. FL denotes full length. B and C, Effects of hyperthermia (HT) on wild type (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 μM, 72 h). E, Inhibition of hyperthermia induced P/R-SMRT interaction by ATRA pretreatment (1 μM, 72 h) in NB4 cells, as determined by Duolink PLA assay. Quantification was performed by image J software with at least 10 cells form each group and normalized to control group. Scale bar is 5 μm. F, Effects of hyperthermia on P/R mutants (located in RARα portion) exogenously expressed in HeLa cells. G, Inhibition of hyperthermia induced exogenous P/R (WT) destabilization by co-silencing of NCoR1 and SMRT in HeLa cells. si-NT represents non-targeting 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 μM, 4h) 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. (<) indicates expected size of the full-length P/R protein, (▲) post-translationally modified, and (<↓) degraded fragments.
Fig. 3. Hyperthermia specifically induces ubiquitin mediated degradation of P/R in a NCoRs 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 induced exogenous P/R (WT) ubiquitination in HeLa cells by simultaneous silencing of NCoR1 and SMRT. si-NT represents non-targeting siRNA; si-N/S represents si-NCoR1 and si-SMRT. C, Effects of hyperthermia on ubiquitination of SMRT, NCoR1 and P/R. HeLa cells were co-transfected with HA-Ub and indicated expression vectors of P/R, pretreated with or without ATRA (2 μM, 4 h) and subjected to hyperthermia prior to IP analysis. EV stands for 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 image J software with at least 10 cells form each group and normalized to control group. Scale bar is 5 μm. 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 h and 12 h. I, Determination of endogenous P/R protein degradation pathway by hyperthermia. NB4 cells were subjected to hyperthermia (HT) and further cultured at 37°C for 12 h with MG132 (0.1 μM) or CQ (10 μM). 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 μg/ml cycloheximide (CHX) for 2 h and subjected to hyperthermia or ATO (2 μM) for 2 h. Then, cells were further cultured at normal condition with (MG132, 10 μM) and/or (chloroquine (CQ), 20 μM) 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 h 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 RIPA insoluble pellet (P) fraction. (*) indicates the IgG band. N, A potential working model of the mechanism by which hyperthermia destabilizes the P/R-NCoRs oncogenic complex.
**Fig. 4. Synergistic effects of hyperthermia with ATO in vitro and in zebrafish model.**

A, Effects of hyperthermia or/and ATO (1 μM) on endogenous P/R protein level in NB4 cells as detected by western blot. Quantification is performed by image J software and presented as mean ± SD (n=3). B, Destabilization of endogenous P/R protein in primary APL cells by hyperthermia or/and ATO (1 μM) for 2 h. Data from samples of 5 different patients was quantified by image J software and presented as mean ± SD. C, Destabilization of endogenous P/R protein stability in NB4 cells by 40°C hyperthermia (HT) or/and 0.5 μM ATO. Quantification is performed by image J software and presented as mean ± SD (n=3). D, Effects of daily hyperthermia or/and ATO on P/R protein level. NB4 cells were subjected to daily hyperthermia (40°C, 0.5 h) and continuous ATO (0.5 μM) 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 μM) and daily hyperthermia (40°C, 1 h/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 DiI as a long-term tracer were subjected to hyperthermia (41°C, 1 h), NB4 or primary blast cells were sorted by FACS and subjected to western blot analysis. G, Synergistic effects of hyperthermia and ATO on NB4 cell proliferation in zebrafish. Zebrafish carrying NB4 xenografts stained with DiI as a long-term tracer were subjected to ATO (1 μM) or/and hyperthermia (41°C, 0.5 h) and further cultured at 37°C for 24 h. NB4 xenografts were visualized by confocal microscope. Cell proliferation rate was calculated by comparing the number DiI positive (red fluorescence) cells from each group with the control group and expressed as mean ± SD from 3 independent assays, n=15 for each group.
Fig. 5. Synergistic effects of hyperthermia with ATO in vitro and in zebrafish model.

A, Clinical course of three relapsed and drug-resistant refractory APL patients who received hyperthermia-ATO combination treatment. Upper panel shows clinical course of the CNS-relapsed APL patient (#I); middle panel shows the clinical course of CNS relapsed APL patient (#II) who developed a chloroma in the brain; lower panel shows the clinical course of the patient (#III) carrying A216V mutation in the unarranged PML allele. BM denotes bone marrow, EM extramedullary, CR complete remission, CNS central nervous system, CSF cerebrospinal fluid, 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 patient (#I) following ATO and daily external hyperthermia [(42°C, 0.5 h)/day] treatment, right panel is quantification from 3 different slides (mean and SD). 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 h)/day] combined with ATO. Positron emission tomography (PET), computer tomography (CT), merged PET/CT as well as 3-dimensional MIP PET (3-D MIP) images are shown. Mean and SD for the standard uptake values (SUV) before and after hyperthermia-ATO combination treatment was calculated by analyzing 4 regions of interests (ROIs; red circles) within the chloroma. The ROIs before treatment were randomly selected and the corresponding post-treatment positions were automatically mapped. SUV\textsubscript{max} values were adjusted according to the background. Paired t-test was used for statistical analysis. The table shows SUV\textsubscript{max} values of the patient. D and E, Destabilization of P/R in PML\textsuperscript{-/-} 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, 4 h), and subjected to ATO or/and hyperthermia treatment. S indicates the supernatant of RIPA lysate and P indicates the insoluble pellet fraction. (\(<\)) indicates expected size of the full-length protein, and (\(<\)) degraded fragments. F, Destabilization of P/R protein in primary APL cells taken from patient (#III) by ATO (1 μM) or/and hyperthermia for 2 h.
Fig. 6. Hyperthermia destabilizes AML1/ETO and TEL/AML1 fusion oncoproteins. A, Confocal microscopy analysis of AML1/ETO’s morphological changes by hyperthermia (42°C, 1 h) in transfected HeLa cells. B and C, Temperature- and time-dependent effects of hyperthermia on AML1/ETO fusion protein stability in transfected HeLa cells. D, Confocal microscopy analysis of TEL/AML1’s morphological changes by hyperthermia (42°C, 1 h) in transfected HeLa cells. E and F, Temperature- and time-dependent effects of hyperthermia on TEL/AML1 fusion protein stability in transfected HeLa cells. S indicates the supernatant of RIPA lysate and P indicates the insoluble pellet fraction.
Figure 6

A

<table>
<thead>
<tr>
<th>AML1/ETO</th>
<th>Merge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con</td>
<td></td>
</tr>
<tr>
<td>HT</td>
<td></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>Temp</th>
<th>AML1/ETO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>43</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>kDa</th>
<th>Flag-AML1/ETO</th>
<th>β-Actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C

<table>
<thead>
<tr>
<th>AML1/ETO</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>kDa</th>
<th>Flag-AML1/ETO</th>
<th>β-Actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

D

<table>
<thead>
<tr>
<th>TEL/AML1</th>
<th>Merge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con</td>
<td></td>
</tr>
<tr>
<td>HT</td>
<td></td>
</tr>
</tbody>
</table>

E

<table>
<thead>
<tr>
<th>Temp</th>
<th>TEL/AML1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>43</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>kDa</th>
<th>Flag-TEL/AML1</th>
<th>β-Actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

F

<table>
<thead>
<tr>
<th>TEL/AML1</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>kDa</th>
<th>Flag-TEL/AML1</th>
<th>β-Actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Hyperthermia Selectively Destabilizes Fusion Oncoproteins
Yasen Maimaitiyiming, Qian Qian Wang, Chang Yang, et al.

Blood Cancer Discov Published OnlineFirst May 11, 2021.

Updated version
Access the most recent version of this article at:
doi: 10.1158/2643-3230.BCD-20-0188

Supplementary Material
Access the most recent supplemental material at:
http://bloodcancerdiscov.aacrjournals.org/content/suppl/2021/04/28/2643-3230.BCD-20-0188.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://bloodcancerdiscov.aacrjournals.org/content/early/2021/04/30/2643-3230.BCD-20-0188. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.