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A New Series of Small Molecular Weight Compounds Induce Read Through of All Three Types of Nonsense Mutations in the *ATM* Gene

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Chemical-induced read through of premature stop codons might be exploited as a potential treatment strategy for genetic disorders caused by nonsense mutations. Despite the promise of this approach, only a few read-through compounds (RTCs) have been discovered to date. These include aminoglycosides (e.g., gentamicin and G418) and nonaminoglycosides (e.g., PTC124 and RTC13). The therapeutic benefits of these RTCs remain to be determined. In an effort to find new RTCs, we screened an additional ~36,000 small molecular weight compounds using a high-throughput screening (HTS) assay that we had previously developed and identified two novel RTCs, GJ071, and GJ072. The activity of these two compounds was confirmed in cells derived from ataxia telangiectasia (A-T) patients with three different types of nonsense mutation in the *ATM* gene. Both compounds showed activity comparable to stop codons (TGA, TAG, and TAA) PTC124 and RTC13. Early structure-activity relationship studies generated eight active analogs of GJ072. Most of those analogs were effective on all three stop codons. GJ071 and GJ072, and some of the GJ072 analogs, appeared to be well tolerated by A-T cells. We also identified another two active RTCs in the primary screen, RTC204 and RTC219, which share a key structural feature with GJ072 and its analogs.

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INTRODUCTION

Approximately 30% of human disease-causing alleles are nonsense mutations.[1] To date, there is no efficient treatment for most genetic disorders. Certain compounds can induce read through of premature termination codons (PTCs), which allows translation of full-length protein. The read through–induced protein is often functional, even when it contains a missense amino acid.[2–5] Thus, chemical-induced read through of stop codons caused by nonsense mutations has great potential as a treatment strategy. This read-through strategy could be especially useful for diseases such as ataxia telangiectasia (A-T) and cystic fibrosis. The severity of these diseases may be significantly reduced by restoring even a small amount of affected protein.[3,6–13]

Despite the promise of this approach, only a few read-through compounds (RTCs) have been described to date. These include aminoglycosides (e.g., gentamicin and G418) and nonaminoglycosides (e.g., PTC124, RTC13, RTC14, and tylosin). Aminoglycosides demonstrate read-through efficiency in many genetic disease models, such as cystic fibrosis,[3,10,14,15] Duchenne muscular dystrophy,[2,16] Hurler syndrome,[17] spinal muscular atrophy,[18] and A-T.[19] However, the therapeutic benefits of aminoglycosides in clinical trials remain unclear.[20–22] The systemic toxicity of most commercial aminoglycosides in mammals further diminishes their clinical potential.[23,24] The macrolide tylosin has RT activity in prokaryotes and is being further evaluated in patients with somatic *APC* mutations in colon cancer.[25] A nonaminoglycoside RTC, PTC124, was developed by high-throughput screening (HTS) using a luciferase-based reporter assay.[13,26] A phase I clinical study in cystic fibrosis showed that PTC124 was well tolerated and more effective than aminoglycosides.[27] However, PTC124's phase 2b clinical study in Duchenne muscular dystrophy patients failed to show significant improvement in the 6-minute walk distance or dystrophin expression.[28] Moreover, PTC124 is not equally effective with all three stop codons, working best on the TGA stop codon.[26] This selective activity would limit the number of patients who might benefit from PTC124. Moreover, PTC124 does not effectively cross the blood–brain barrier, a critical factor for treating neurological disorders such as A-T, Alzheimer diseases, and the CNS effects encountered in Hurler patients. Therefore, successfully developing new RTCs with optimized efficacy and low toxicity holds great promise for numerous genetic diseases that are caused by nonsense mutations. Furthermore, the potential therapeutic benefit of a small molecular read-through (SMRT) compound may also extend to cancer-prone individuals carrying highly penetrant nonsense mutations in genes, such as *BRCA1*, *BRCA2*, and *CHEK2*, if such medications were proven

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safe for long-term prophylactic therapy, and cancer-preventing benefits could be convincingly documented.

In an effort to develop novel RTCs, we previously described two SMRT compounds, RTC13 and RTC14, that can read through nonsense mutations in both the *ATM* and *dystrophin* genes.[6] We further demonstrated that RTC13 has *in vivo* read-through activity in *mdx* mice (a Duchenne muscular dystrophy model carrying a TAA nonsense mutation in exon23 of the *dystrophin* gene) that induces dystrophin protein.[12] Recently, we were able to generate a series of active RTC13 analogs that are currently being characterized in different animal models with nonsense mutations.[29] Herein, we report a second series of SMRT chemicals, exemplified by GJ071 and GJ072, that are comparable to or better than RTC13 or PTC124. We further describe two additional compounds identified in our primary screen, RTC204 and RTC219, that share a key structural element and read-through activity with GJ072 and its analogs.

RESULTS

HTS identified two novel small molecular weight RTCs

We previously developed a luciferase-independent HTS assay, protein transcription-translation (PTT)–enzyme-linked immunosorbent assay (ELISA), that allowed us to screen small molecular weight libraries.[6] The PTT-ELISA was based on an *in vitro* transcription and translation system driven by a plasmid containing "region 5" of the *ATM* gene (coding sequence for 403-1986 aa) with a TGA C mutation (c.5623C>T; Arg1875X). This assay has proven very reliable in that most "hits" previously selected by the assay have had RT activity. In this study, we screened ~36,000 additional compounds and were able to identify two novel active parent compounds, GJ071 and GJ072. Both compounds have structures different from any previously reported RTCs (structures shown in **Figure 1a**). Their *in vitro* read-through activity on a TGA mutation was subsequently confirmed by PTT-ELISA in dose-response experiments (**Figure 1b**). This activity was again confirmed in tertiary assays with A-T cells carrying each of the three types of nonsense mutations (see below).

RTCs induced read through of different PTCs in A-T cells

To characterize the cellular read-through activity of GJ071 and GJ072, we selected three A-T lymphoblastoid cell lines derived from patients containing different types of homozygous *ATM* nonsense mutations: AT153LA (c.8977C>T, TGA A, Arg2993X), AT229LA (c.3102T>G, TAG A, Tyr1034X), and AT187LA (c.5908C>T, TAA G, Gln1970X). The A-T cells were treated with each RTC for 4 days and then harvested to measure ATM kinase activity, which is often completely deficient in A-T cells. Both GJ071 (**Figure 2a**) and GJ072 (**Figure 2b**) induced ATM kinase on both TGA and TAG stop codons and restored ATMpSer1981 autophosphorylation and SMC1pSer966 transphosphorylation as measured by FACS. Notably, GJ071 and GJ072 were also active in A-T cells with a homozygous TAA mutation (a stop codon that was previously reported to be more difficult to read through than TGA and TAG) and induced ATMpSer1981 foci formation after radiation (IRIF) (**Figure 3a** and **3b**). Furthermore, both compounds were also able to induce detectable full-length ATM protein in treated A-T cells, as demonstrated by ATM-ELISA (**Figure 3c**).

Since a hallmark of A-T is the hypersensitivity of cells to ionizing radiation, we further assessed whether GJ071 and GJ072 could abrogate cellular radiosensitivity in A-T cells. AT242LA cells with two heterozygous stop codons, TGA (7096G>T[a], E2366X and TAG

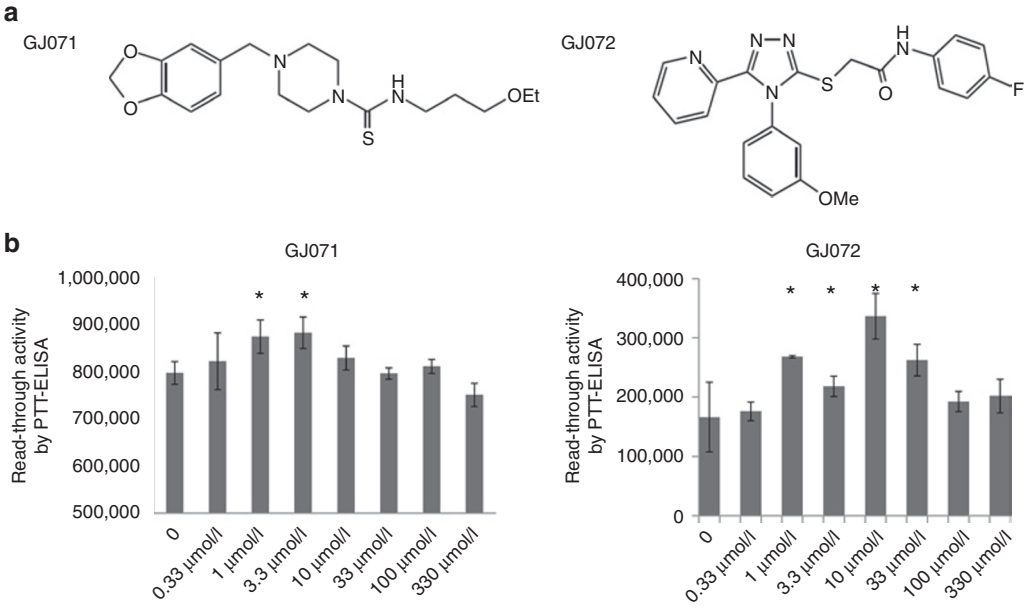

Figure 1 HTS of chemical libraries identified two novel SMRT compounds, GJ071 and GJ072. (**a**) Chemical structures of GJ071 and GJ072. (**b**) *In vitro* read-through activity was measured by PTT-ELISA using a plasmid containing a disease-causing *ATM* mutation, 5623C>T (TGA C). The compounds were tested independently. G418 was used as positive control for each experiment (not shown). The experiments were repeated multiple times; a representative experiment is shown here and raw RLU numbers are used. *$P \leq 0.05$ as compared with untreated control. A-T, ataxia telangiectasia; SMRT, small molecular read through.

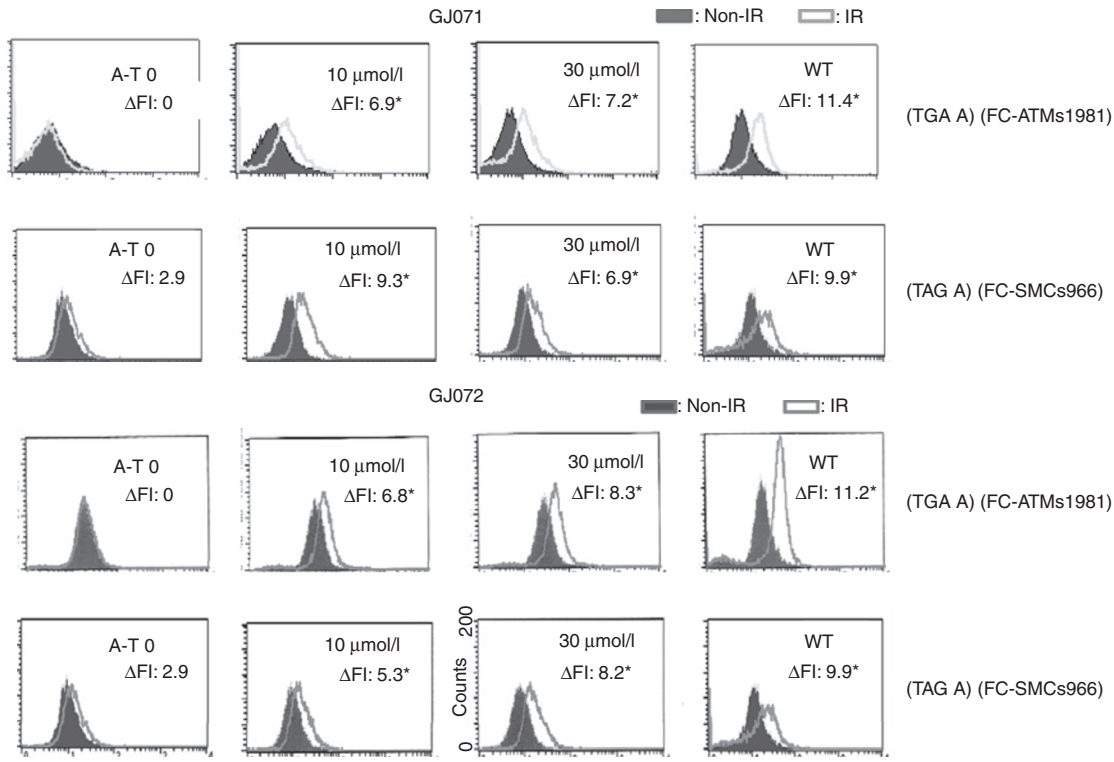

Figure 2 GJ071 and GJ072 induced ATM kinase activity in A-T cells carrying homozygous TGA or TAG stop codons. A-T cells were treated with each compound for 4 days before harvesting. The ATM kinase activity in AT153LA cells (TGA A) was measured by FC-based ATMs1981 autophosphorylation (FC-ATMs1981), and the ATM kinase activity in AT229LA cells (TAG A) was measured by FC-based SMC1s966 transphosphorylation (FC-SMC1s966). The experiments were repeated multiple times; representative experiments for FC-ATMs1981 and FC-SMC1s966 are shown here. A right shift of the fluorescence intensity peaks (ΔFI) indicated the restored ATM kinase activity by both compounds (top: GJ071; bottom: GJ072). *$P \leq 0.05$ compared with nontreated A-T cells. A-T, ataxia telangiectasia.

(7913G>A[b], W2638X), were treated with each compound and followed by colony survival assay. Both GJ071 and GJ072 improved AT242LA cell radiosensitivity from the "radiosensitive" range (<21 SF%) to the "intermediate" range (21–36 SF%) (**Supplementary Figure S1**); these ranges are currently used for A-T clinical diagnosis.[30]

Structure-activity relationship studies used to derive active analogs of GJ072

To establish a structure-activity relationship and improve design of novel SMRT compounds, we synthesized and tested analogs of both GJ071 and GJ072. Each analog was tested at 10 and 30 μmol/l. For GJ071, none of the six analogs of GJ071 showed significant read-through activity (data not shown). In contrast, eight of the 11 GJ072 analogs induced ATM kinase activity in A-T cells carrying different homozygous nonsense mutations; their chemical structures are shown in **Figure 4**. The activity of these GJ072 active analogs in TGA-containing A-T cells was demonstrated by increased FCATMpSer1981 levels, indicated by "ΔFI" (fluorescence intensity difference between paired non-irradiation (IR) and IR samples) (**Figure 5**), as well as by IR-induced ATMs1981 foci formation assay (IRIF) (some analogs shown in **Supplementary Figure S2**). Similar to the parent compound GJ072, these analogs were also active in A-T cells containing TAG or TAA stop codons. The activity of the analogs on TAA PTC (in AT187LA cells) was demonstrated by both the FCATMpSer1981 assay (**Figure 6**) and ATMpSer1981-IRIF assay (**Supplementary Figure S3**). The activity of the analogs on a TAG stop codon (AT229LA cells) is shown in **Supplementary Figures S4** and **S5**. Notably, some GJ072 analogs (e.g., GJ103, GJ106, GJ109, and GJ111) consistently demonstrated their activities in all three PTCs by both FCATMpSer1981 (**Figures 5** and **6**, **Supplementary Figure S4b**) and IRIF assays (**Supplementary Figures S2 and S5**), implying their potential for further development. To date, we have developed a water soluble salt form (GJ103-salt) for one of these analogs, GJ103, which will enable us to evaluate its *in vivo* activity by systematic administration.

Interestingly, we also identified two other active compounds in the primary screen, RTC204 and RTC219; both share a key structural feature with GJ072 and its analogs. In particular, RTC219 has the 3-(amidomethylthio)1,2,4-triazole unit of the earlier compounds but with different substituents at positions 4 and 5, while RTC204 has a somewhat related 5-(acylhydrazidomethylthio)tetrazole unit. The structures and activities of RTC204 and RTC219 in A-T cells are shown in **Figure 7**.

Comparison of GJ071 and GJ072 with other known RTCs

We next compared the efficacy of GJ071 and GJ072 with two known RTCs, RTC13, and PTC124.[6,26] We also included an active analog of GJ072, GJ103, since its acid functionality could be converted to a water soluble form (GJ103-salt) for further evaluation. As shown in **Figure 8**, GJ072 and its analog GJ103 showed a similar activity to RTC13 and were arguably better than PTC124 in

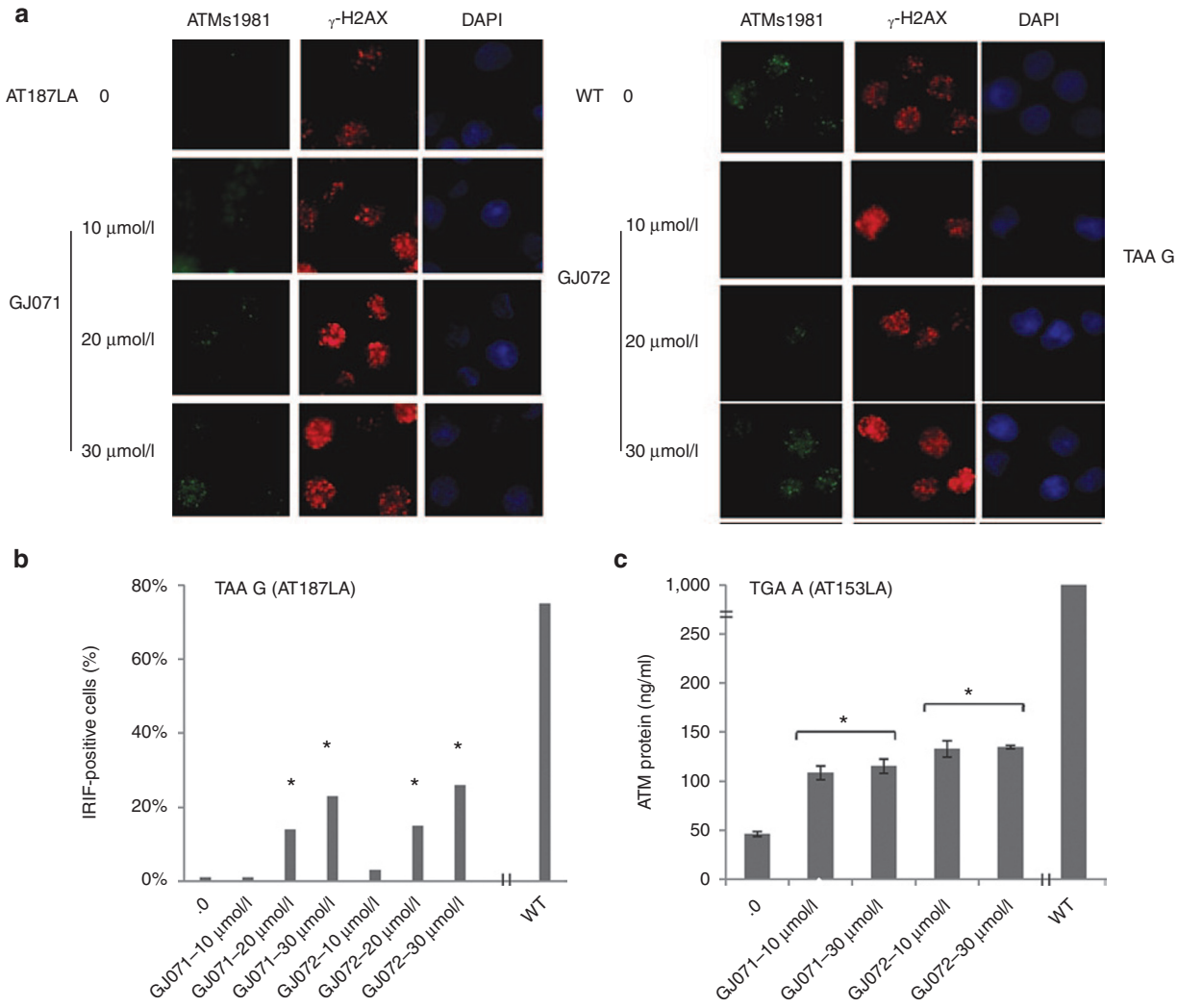

Figure 3 GJ071 and GJ072 induced ATMs1981 foci formation in A-T cells with a TAA mutation; full-length ATM protein levels were abrogated in A-T cells with a TGA mutation. A-T cells were treated with each compound for 4 days prior to testing for read-through effects. Wild-type cells were used as positive controls. (**a**) RTC-induced ATM1981 foci in AT187LA cells with a homozygous TAA mutation. The experiments were repeated multiple times; a representative experiment is used here to show ATMs1981 foci formation after 2-Gy radiation. The formation of γ-H2AX foci was included as a control for effective radiation damage; (**b**) Quantification of **Figure 3a** for IRIF-positive cells percentage; >120 cells were counted for each sample; (**c**) RTC-induced ATM protein in AT153LA cells with TGA mutation measured by ATM-ELISA. *$P \leq 0.05$, compared with nontreated A-T cells. A-T, ataxia telangiectasia; RTC, read-through compounds.

A-T cells (AT153LA, TGA A). Moreover, GJ071, GJ072, and some analogs of GJ072 (such as GJ103 and GJ105) did not show obvious cytotoxicity in A-T cells at concentration as high as 300 μmol/l (data not shown), suggesting that the new series of RTCs reported here may have greater potential for drug development.

DISCUSSION

In this study, we identified and characterized the read-through activity of two new groups of SMRT compounds, GJ071 and GJ072, and their analogs. This expands our experience to four parent SMRT groups: RTC13, RTC14, GJ071, and GJ072. GJ072 and RTC13 share a superficial similarity with previously described PTC124 in terms of their three-ring structures, while GJ071 and RTC14 have very different structures. Our data showed that GJ071 and GJ072 and some of their analogs (such as GJ103) had similar read-through activity as RTC13 or RTC14, but were more tolerable than RTC13 and RTC14 to A-T cells. The concentrations (10 and 30 μmol/l) we used for all analogs were based on the optimum range for the parent compounds; for the parent compounds, we did not see significant improvement of response in A-T cells at higher concentrations (data not shown). Importantly, similar to RTC13 and RTC14, these novel RTCs were active for all three premature stop codons in A-T cells. Considering the limited market potential for SMRT drugs on rare genetic diseases, the "one-drug-fits-all" model would be a distinct advantage for translational potential.

Most of the active analogs of GJ072 had low cLogP values, which is predictive of good absorption or permeation potential *in vivo*. In addition, the GJ103 salt form is water soluble, making it much easier to work with in *in vivo* experiments. Importantly, we found that two additional compounds identified in this primary screen, RTC204 and RTC219, shared a similar structural feature with GJ072 and its analogs, implying that this structural feature

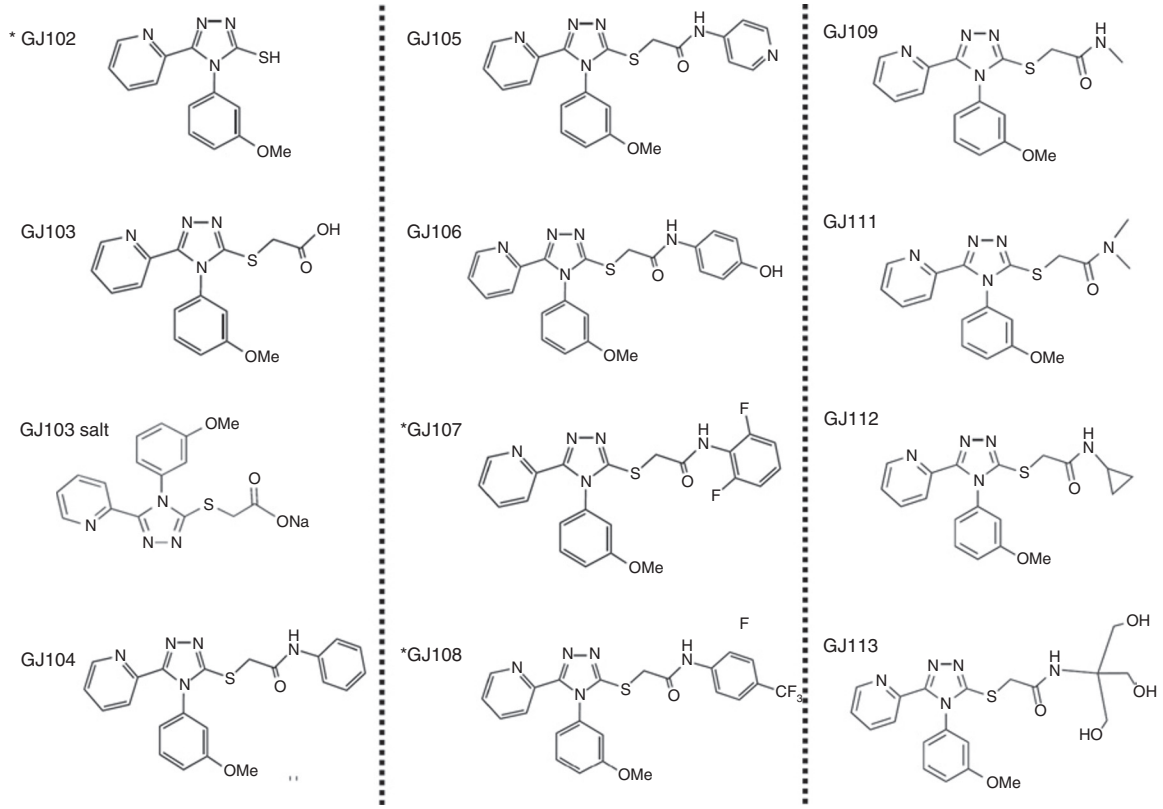

Figure 4 Structures of synthesized GJ072 analogs. The synthesis of the parent compounds and their analogs is described in supplemental materials (see compound preparation). All were active except GJ102, GJ107, and GJ108. *Inactive; see text and **Figure 5**.

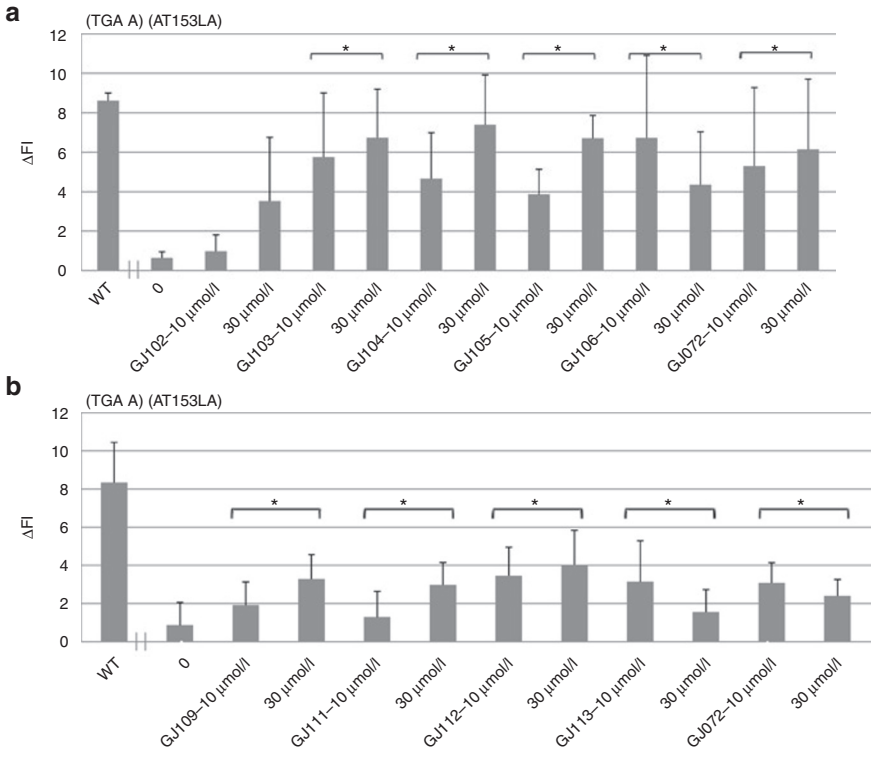

Figure 5 The effect of GJ072 analogs on TGA PTC in A-T cells measured by FC-ATMs1981. AT153LA cells with a homozygous TGA mutation were exposed to RTCs for 4 days before assaying. Restored ATM kinase activity was indicated by increased fluorescence intensity measured by flow cytometry (ΔFI). (**a**) Activity of GJ102, GJ103, GJ104, GJ105 and GJ106. The data shown were summarized from four independent experiments; (**b**) Activity of GJ109, GJ111, GJ112 and GJ113, the data shown were summarized from four independent experiments. *$P \leq 0.05$ compared with nontreated A-T cells. A-T, ataxia telangiectasia; RTC, read-through compounds.

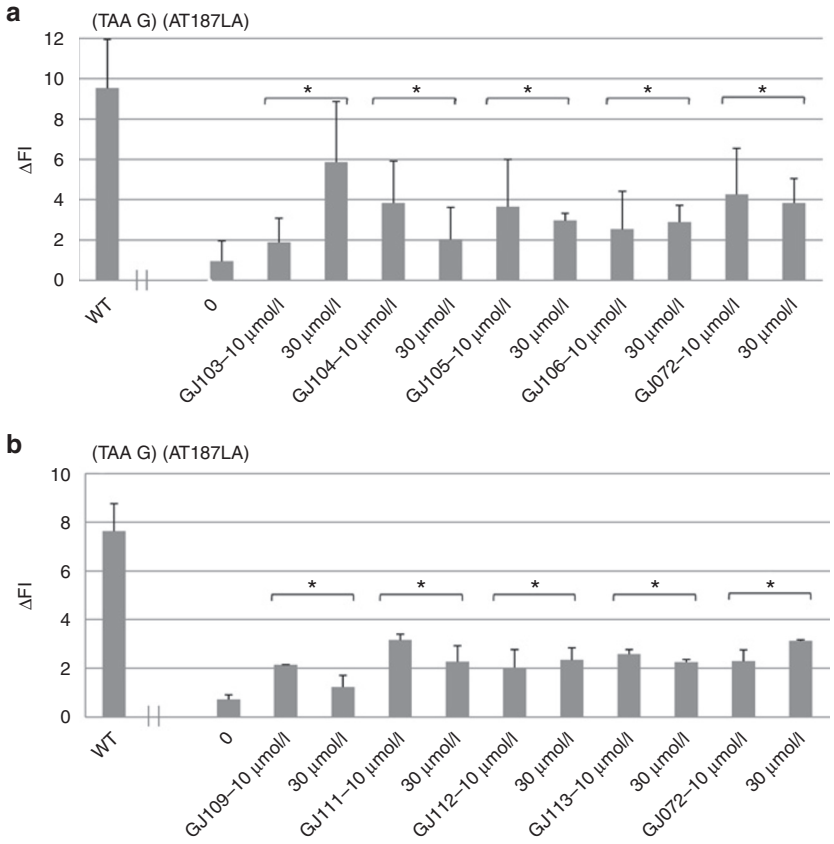

Figure 6 The effect of GJ072 analogs on TAA in A-T cells measured by FC-ATMs1981. AT187LA cells with homozygous TAA mutation were exposed to RTCs for 4 days before assaying. Restored ATM kinase activity was indicated by increased "ΔFI." (**a**) Activity of GJ103, GJ104, GJ105, and GJ106; (**b**) Activity of GJ109, GJ111, GJ112, and GJ113. Data shown in (**a**) and (**b**) were derived from three independent experiments, respectively. *$P \leq 0.05$ as compared with nontreated A-T cells. A-T, ataxia telangiectasia.

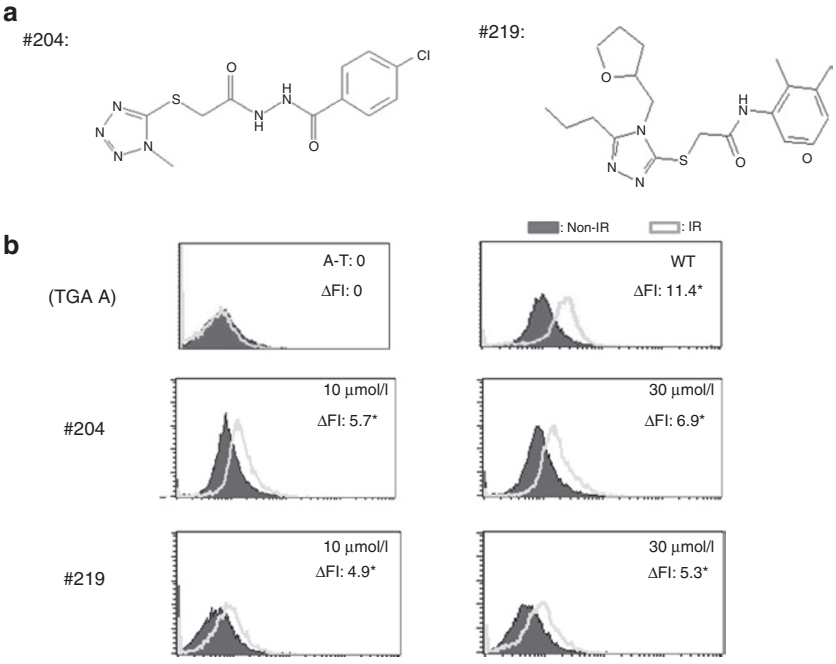

Figure 7 GJ204 and GJ219 identified from primary screen shared a similar structural feature with GJ072. (**a**) Chemical structures of RTC204 and RTC219; (**b**) RTC204 and RTC219 restored ATMs1981 autophosphorylation in AT153LA cells with homozygous TGA A mutation after a 4-day treatment (indicated by the right shift of fluorescence peak). The experiments were repeated multiple times; a representative experiment is used to show histogram change of fluorescence intensity. *$P \leq 0.05$ as compared with nontreated A-T cells. A-T, ataxia telangiectasia.

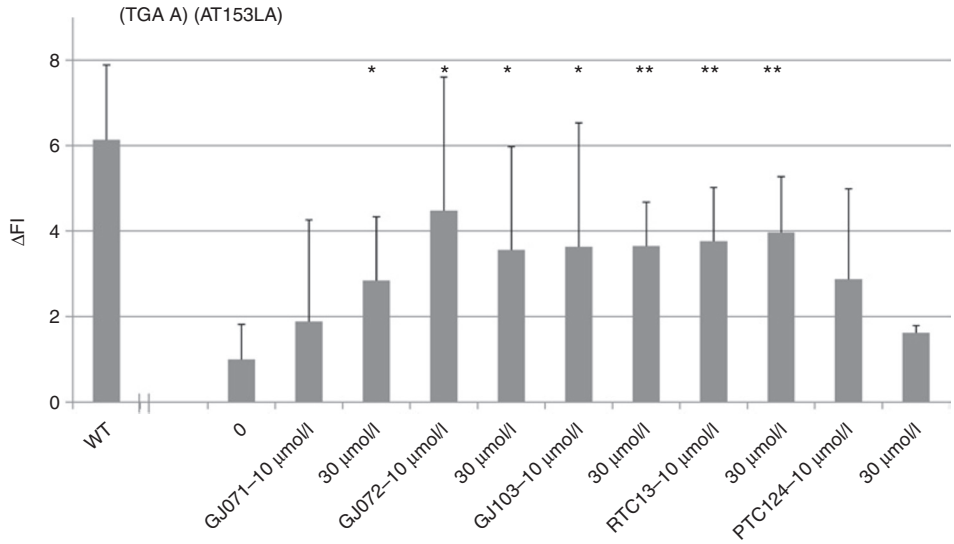

Figure 8 Read-through activity comparison of GJ071 and GJ072 with other known RTCs in A-T cells. AT153LA cells with homozygous TGA mutation were exposed to GJ071, GJ072, GJ103, RTC13, and PTC124 for 4 days, respectively, and ATM kinase activity was measured by FC-ATMs1981. Restored ATM kinase activity was indicated by "ΔFI." Data shown were derived from three independent experiments. *$P \leq 0.05$; **$P \leq 0.01$ as compared with nontreated A-T control. A-T, ataxia telangiectasia; RTC, read-through compounds.

may be responsible for their read-through activity. Since all of the known RTCs are thought to function by interfering with parts of the ribosome,[31,32] the potential ribosomal interactions of these new SMRT compounds need further attention.

The influence of nonsense-mediated decay on PTC read-through strategy should be considered since mRNAs carrying nonsense mutations are degraded by this pathway[33,34] and inhibition of nonsense-mediated decay may, therefore, stabilize mutant mRNA transcripts and increase RTC-induced read-through output.[35] These mutations may also be missed, if sequencing is performed on cDNA derived from patient cells without first inhibiting nonsense-mediated decay. Other major issues related to read through in A-T patients that require further studies include (i) the potential risk of triggering inappropriate "retroactive" apoptosis after restoring ATM protein to A-T cells,[36] (ii) the requirement that ATM induction occurs in cerebellar cells, that is, a SMRT drug's ability to cross the blood–brain barrier,[37,38] and (iii) the potential side effects of these compounds on normal stop codons. It also remains possible that substituting a "false" amino acid for a premature stop codon in ATM protein, especially in the key functional domains, may disrupt normal function. To date, we have preliminary data to suggest that many of our SMRT drugs will reach the cerebellum, a key site for reversing the neuropathology of A-T. The possibility of read through of normal stop codons by other RTCs has been investigated in numerous studies, but none have described read through of the final normal stop codon in the last exon. We are presently evaluating the activity and potential toxicities of a lead SMRT compound on AT-derived neural progenitor cells.[39] Further development of these compounds may eventually contribute to the identification of a drug candidate with improved pharmacokinetic properties and minimal off-target toxicity for genetic diseases caused by nonsense mutations.

MATERIALS AND METHODS

Lymphoblastoid cells. Different A-T Lymphoblastoid cells were used to assess ATM functions and cytotoxicity of RTCs. These include AT153LA, AT229LA, AT187LA, and AT242LA. All cells were derived from fresh blood samples from our laboratory, following which they were anonymized in accordance with Institutional Review Board guidance.

Chemical libraries and analogs preparation. Chemical libraries screened were provided by Molecular Shared Screening Resources, California NanoSystems Institute, UCLA. All the analogs were prepared according the protocols in Supplementary Materials.

PTT-ELISA and HTS. The HTS was based on a cell-free PTT-ELISA system that was developed in our laboratory.[6,40] Approximately 36,000 compounds were screened to identify novel RTCs. Each compound was screened at a final concentration of 10 μM. Screening was performed on a fully integrated CORE System (Beckman Coulter-SAGIAN, Indianapolis, IN).

Flow cytometry analysis of ATMpSer1981 and SMC1pSer966 phosphorylation. FCpSerSMC1 and FCATMpSer1981 assay was performed as previously described.[6,41] In brief, cells were resuspended in PBS and radiated for 10 Gy. After 1 hour, the cells were fixed and permeabilized using the FIX and PERM cell permeabilization kit (Invitrogen, Grand Island, NY). The cells were then incubated with ATMpSer1981 antibody or SMC1pSer966 antibody for 2 hours at room temperature. Cells were washed and stained with Alexo488-anti-mouse IgG (Invitrogen) for 45 minutes. Cells were then washed, resuspended in PBS with 0.2% paraformaldehyde, and analyzed using FACScan (BD Biosciences, San Jose, CA).

Immunofluorescence of ATMpSer1981-IRIF. Immunostaining of ATMpSer1981 nuclear foci was performed as previously described.[6,42] Briefly, cells were irradiated with 2 Gy after a 4-day RTC exposure period and incubated at 37 °C for 30 minute. The cells were dropped onto coverslips, fixed with paraformaldehyde, and permeabilized. The coverslips were then blocked with PBS with 10% FBS and incubated with mouse anti-ATM pSer1981 for 1 hour at RT (Rockland Immunochemicals, Gilbertsville, PA). Cells were washed and blocked again for 1 hour and then stained with FITC-conjugated anti-mouse IgG (1:150; Jackson ImmunoResearch, West Grove, PA) and mounted onto slides.

Immunoassay for measurement of intranuclear ATM protein. Read through-induced full-length ATM protein in A-T cells was measured by ATM-ELISA immunoassay (Butch *et al*. 2004). Cell nuclear extracts were prepared using NE-PER protocol (Pierce, Rockford, IL). Then,

ATM-ELISA was performed using 200 µg of nuclear extracts. ATM concentrations of tested samples were calculated from the standard calibration curve, using purified ATM protein.

Colony survival assay. Colony survival assay was performed as previously described.[30,43] A-T cells were treated and plated in duplicate in 96-well plates at 100 and 200 cells per well, respectively. One of the duplicate plates was exposed to 1.0 Gy radiation, whereas the other one was not irradiated. The cells were incubated for 10–13 days and then stained with MTT.

Cytotoxicity by XTT assay. Cytotoxicity was measured by Cell Proliferation Kit II (Roche Applied Science, Indianapolis, IN). Briefly, cells were seeded into a flat-bottom 96-well plate, including control wells containing complete growth medium alone as blank absorbance readings. After RTC treatment, activated-XTT Solution was added into each well, and the cells were returned to the cell culture incubator for 12–14 hours. The absorbance was measured at 480 nmol/l with relevant 630 nmol/l to assess nonspecific readings.

SUPPLEMENTARY MATERIAL

Figure S1. GJ071 and GJ072 abrogated the radiosensitivity of A-T LCLs.

Figure S2. Active GJ072 analogs induced ATMs1981 foci in AT153LA cells with TGA PTC.

Figure S3. Active GJ072 analogs induced ATMs1981 foci in AT187LA cells with TAA PTC.

Figure S4. GJ072 analogs induced read through of TAG PTC in A-T cells.

Figure S5. Active GJ072 analogs induced ATMs1981 foci in AT229LA cells with TAG PTC.

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