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Synthesis and evaluation of compounds that induce readthrough of premature termination codons

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ABSTRACT

A structure–activity relationship (SAR) study was carried out to identify novel, small molecular weight compounds which induce readthrough of premature termination codons. In particular, analogs of RTC13, **1**, were evaluated. In addition, hypothesizing that these compounds exhibit their activity by binding to the ribosome, we prepared the hybrid analogs **13** containing pyrimidine bases and these also showed good readthrough activity.

A nonsense mutation is a point mutation in a sequence of DNA that results in a premature termination codon (PTC) or a nonsense codon in the transcribed mRNA.[1] These cause the formation of either no protein or of a truncated, unstable, nonfunctional protein. Approximately 30% of common genetic diseases result from nonsense mutations,[2] such as in Ataxia-telangiectasia (A-T), Duchenne muscular dystrophy (DMD), cystic fibrosis (CF), and spinal muscular dystrophy (SMA).[3] A-T is a rare autosomal recessive neurodegenerative disease that affects primarily the cerebellum and causes truncal ataxia.[4] A-T patients also suffer from an increased risk of cancer, immunodeficiency and hypersensitivity to ionizing radiation and some chemotherapeutics, such as alkylating agents. Mutations in the *ATM* (Ataxia-Telangiectasia Mutated) gene cause A-T. ATM is a 370 kD 'high molecular weight' protein kinase that is found in all cells, primarily in the nucleus. It phosphorylates over 700 downstream protein targets, which are involved primarily in the recognition and repair of DNA double strand breaks but also in responding to oxidative stress, cell cycle checkpoints, and nonsense mediated decay. Our previous efforts[3a,5] have been directed towards the development of drugs for A-T with either antisense morpholino oligonucleotides (AMO) or aminoglycoside readthrough compounds (RTCs). Delivery has been a major obstacle in bringing AMOs to therapeutic fruition. RTCs like gentamycin, an aminoglycoside,[6] have undesirable off-target effects, such as nephrotoxicity and hearing loss.[7] Furthermore, because the cerebellum is the major site of pathology in A-T[8] and aminoglycosides are too large to cross the blood–brain barrier, we sought to develop more specific and potent small molecules. We report herein a structure–activity relationship study, which has identified novel small molecular weight nonaminoglycoside readthrough compounds.

For the purpose of finding novel readthrough compounds, we recently[9] reported a high-throughput screen (HTS) of 34,000 compounds using a PTT-ELISA assay that resulted in the identification of 12 low molecular weight compounds with PTC readthrough activity. From these, two potent lead compounds, RTC13 **1** and RTC14 **2**, (Fig. 1) consistently induced functional ATM protein in ATM-deficient cells containing disease-causing nonsense mutations, as demonstrated by direct measurement of ATM protein, restored ATM kinase activity, and colony survival assays for cellular radiosensitivity. We exposed A-T lymphoblastoid cell lines (LCLs) to each RTC compound for 4 days prior to harvesting the cells.[9] Although **2** showed a slightly higher in vitro readthrough signal than **1**, **1** was less cytotoxic to LCLs.[9] Therefore we concentrated our SAR efforts on analogs of **1**. Herein, we report the synthesis and evaluation of several 2-imino and 2-thioxothiazolidin-4-one derivatives with regard to their ability to translate through the premature termination codons directly caused by nonsense mutations.

We examined four structural changes in **1** (Fig. 2), namely: change in the heteroatom of the 2-carbonyl unit of the thiazolidin-4-one (A); variation of the aryl group on the furan ring (B); introduction of an alkyl group on the ring nitrogen of the thiazoli-

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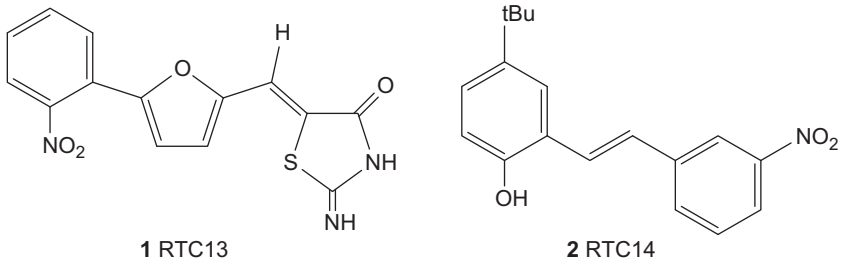


Figure 1. Structures of HTS hits RTC13 **1** and RTC14 **2**.

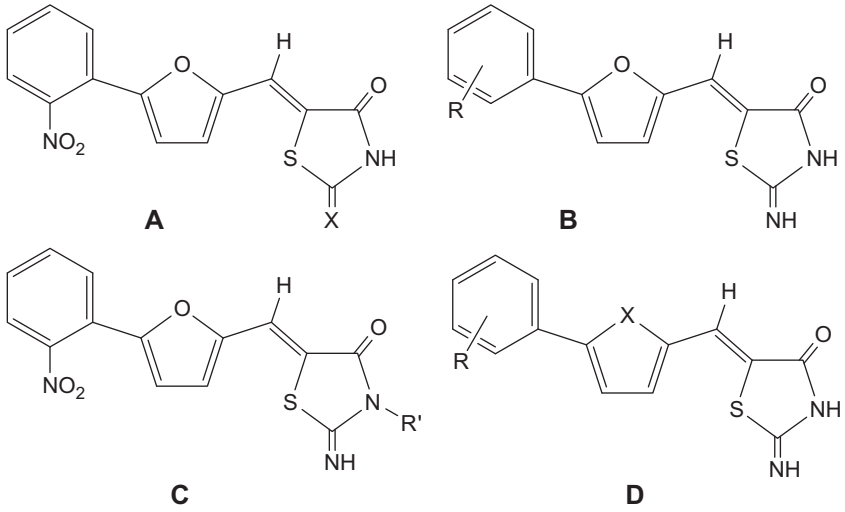
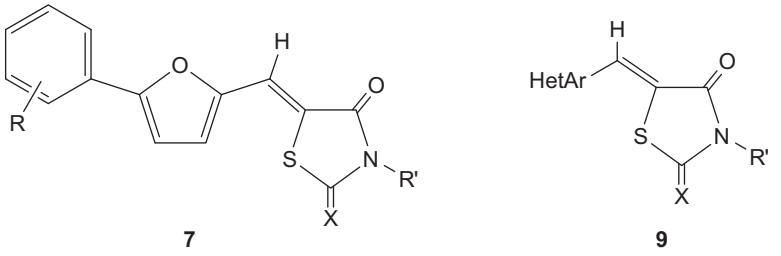


Figure 2. Four structural variations of lead compound RTC13 **1**.

Table 1
The evaluation of readthrough activity of compounds **7**



Entry	Compd	X	R	R′	FC-ATM (Delta-FI, 30 μM)
1	RTC13	NH	2-NO_2	H	3.27
2	**7a**	NH	H	H	Negative
3	**7b**	S	2-NO_2	H	Negative
4	**7c**	NH	2-NO_2	Me	Negative
5	**7d**	NH	2-CF_3	H	Negative
6	**7e**	NH	2-OMe	H	Negative
7	**7f**	NH	2-Cl	H	Negative
8	**7g**	NH	2-F	H	Negative
9	**7h**	NH	3-MeO	H	1.90
10	**7i**	NH	3-Cl	H	2.27
11	**7j**	NH	3-F	H	Negative
12	**7k**	S	2-CF_3	H	Negative
13	**7l**	S	2-MeO	H	Negative
14	**7m**	S	2-Cl	H	3.55
15	**7n**	S	2-F	H	2.86
16	**7o**	S	3-OMe	H	2.66
17	**7p**	S	3-Cl	H	2.86
18	**7q**	S	3-F	H	3.44

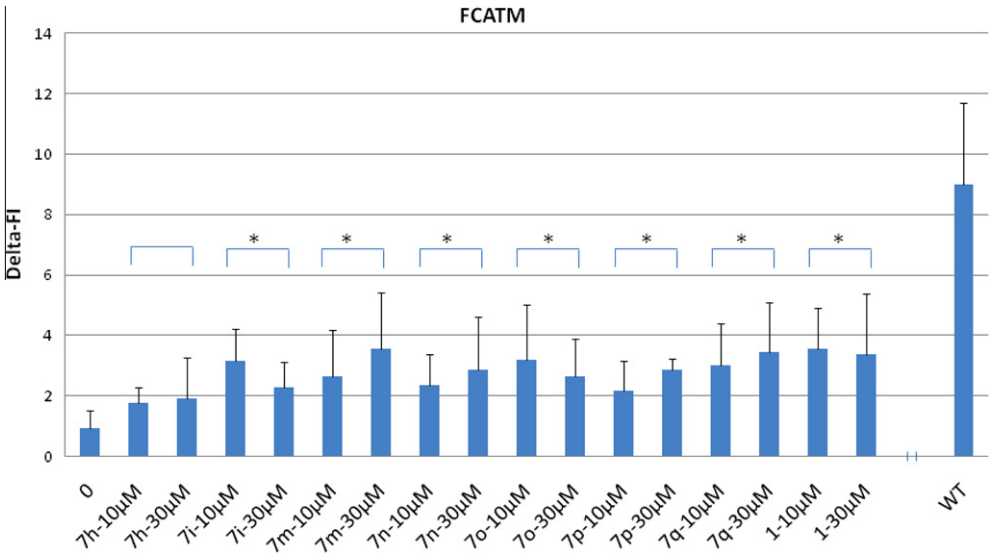
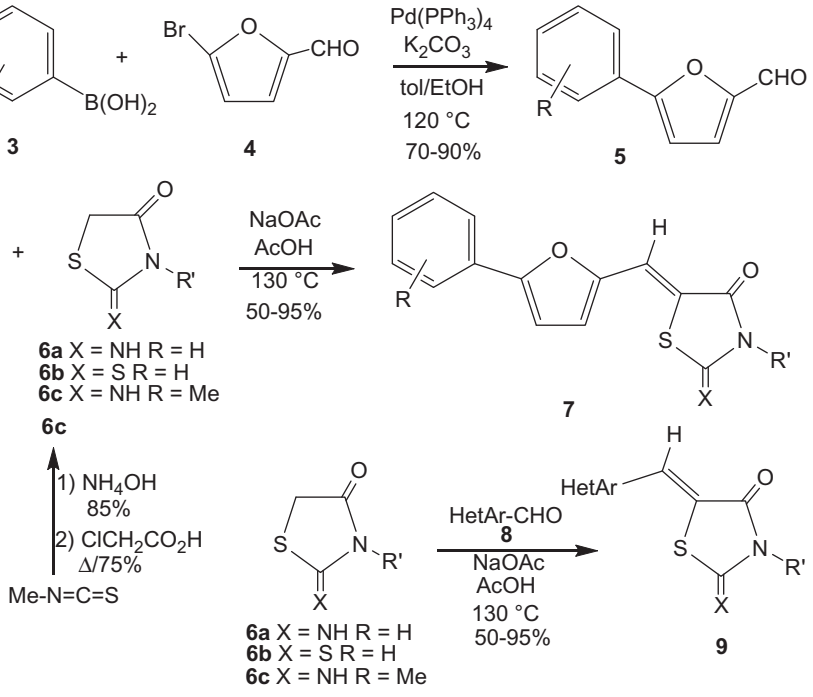


Figure 3. Measurement of readthrough activity of compounds **7** using FC-ATMs1981 in A-T cells. After 4 days of compound treatment, A-T cells were irradiated for 10 Gy and collected for assaying. Wild type cells (WT) were used as a positive assay control. The data from 3 sets of independent of experiments are summarized here. The increased Delta-FI (difference of fluorescent intensity before and after IR) indicated the restoration of ATM autophosphorylation at Serine 1981. All RTC-treated cell samples showed significant differences as compared to untreated cells (0), except **7h** (*p <0.05, by t-tests comparing paired concentrations of treated to untreated cells).

Table 2
The evaluation of readthrough activity of compound **9**

Entry	Compd	HetAr	R′	FC-ATM (Delta-FI, 30 μM)
1	**9a**	2-Benzofuranyl	NH	Negative
2	**9b**	2-Indolyl	NH	Negative
3	**9c**	3-Indolyl	NH	Negative
4	**9d**	5-(Pyridin-2-yl)-furan-2-yl	NH	Negative
5	**9e**	5-Phenylthiophen-2-yl	NH	Negative
6	**9f**	Furan-2-yl	NH	Negative
7	**9g**	2-Benzofuranyl	S	3.50
8	**9h**	2-Indolyl	S	Negative
9	**9i**	3-Indolyl	S	Negative
10	**9j**	5-(Pyridin-2-yl)-furan-2-yl	S	Negative



Scheme 1. Synthesis of compounds **7** and **9**.

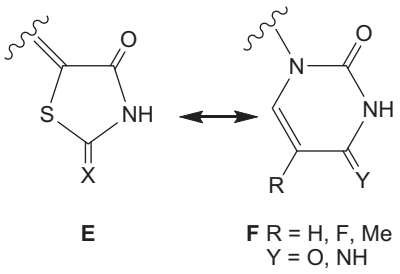


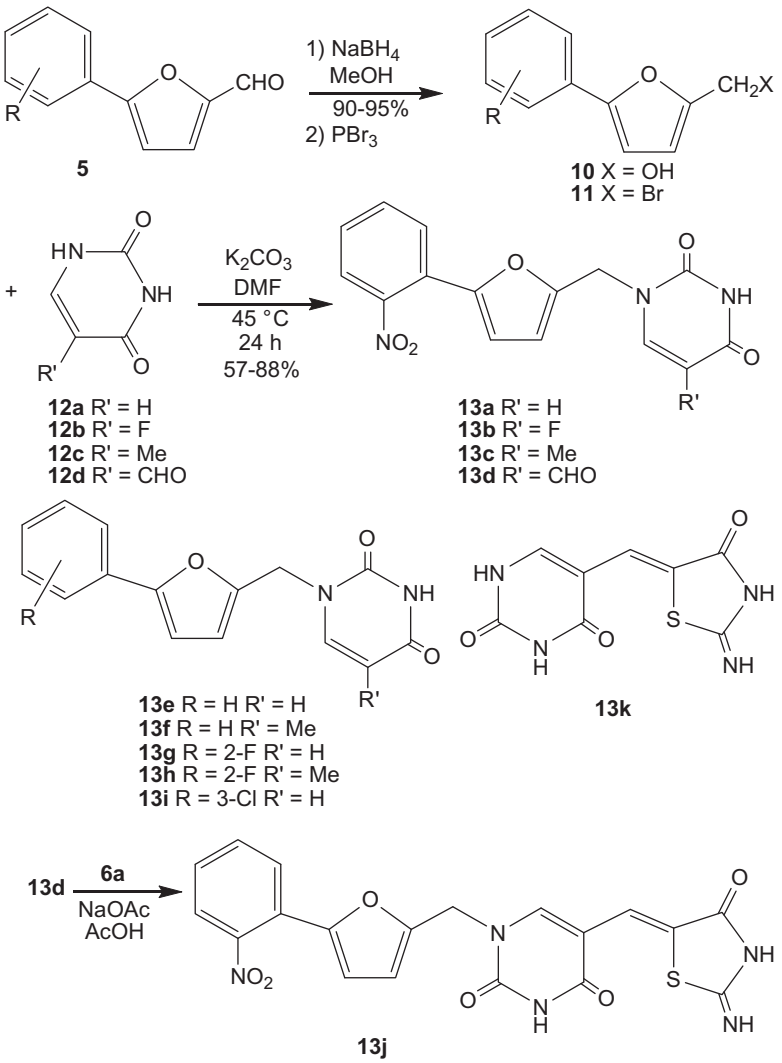
Figure 4. Change of thiazolidinone unit to pyrimidinone.

din-4-one (C); and introduction of different aryl groups as the central ring unit (D).

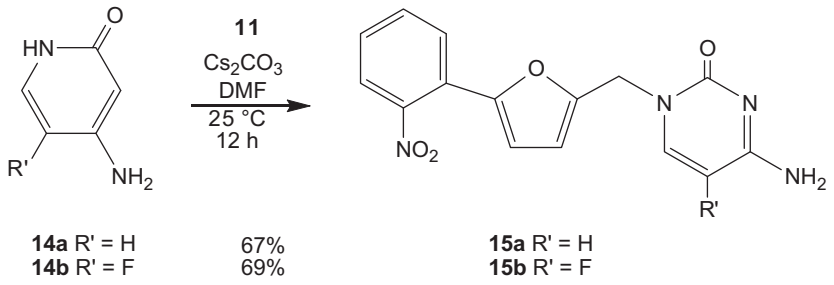
The syntheses of the compounds listed in Tables 1 and 2 are shown in Scheme 1.[10] Coupling of the arylboronic acids **3** with commercially available 5-bromofurfural **4** afforded the 5-arylfurfurals **5** in good yields. Condensation of these compounds with any of the thiazolidinones **6a–c** furnished the desired products **7** in 50–95% yield. The two thiazolidinones **6a** and **b** were commercially available and the *N*-methyl analog **6c** was prepared in two steps from methyl isothiocyanate as shown. Also substitution of any of several heteroaryl aldehydes **8** for the 5-arylfurfural allowed the synthesis of the heteroarylmethylene analogs **9**. Most of the heteroaryl aldehydes **8** were commercially available while those that were not, for example, the benzofuran-2-, indole-2-, 5-phenylthiophene-2-, and 5-(2-pyridyl)-furan-2-carboxaldehydes, were prepared by straightforward routes (see Supplementary data).

These two series, **7** and **9**, were tested for readthrough activity using an assay in which the ATM protein kinase activity was measured using a flow cytometry-based ATMs1981 autophosphorylation assay (FC-ATM).[11] ATM kinase activity is demonstrated by the change in the fluorescence intensity before and after ionizing radiation (Delta-FI) (Fig. 3). An increased Delta-FI indicates the restoration of ATM kinase activity by a compound. Table 1 shows the data for the analogs **7** in which the groups X, R, and R′ were varied. Several analogs showed reasonably good readthrough activity, with the 2-chlorophenyl and the 3-fluorophenyl 2-thioxo analogs **7m** and **q** being the best of this group. Among the heteroaryl analogs **9**, only the 2-benzofuranyl 2-thioxo analog, **9g**, gave positive results (Table 2).

We hypothesized that the mechanism of action of these novel tricyclic aromatic compounds might be similar to that of the aminoglycosides, which have been reported to exert their readthrough effects through an interaction with the ribosome.[12] In particular we wondered whether the 2-imino and 2-thioxothiazolidin-4-one units **E** might be binding to nucleobases in the ribosome via

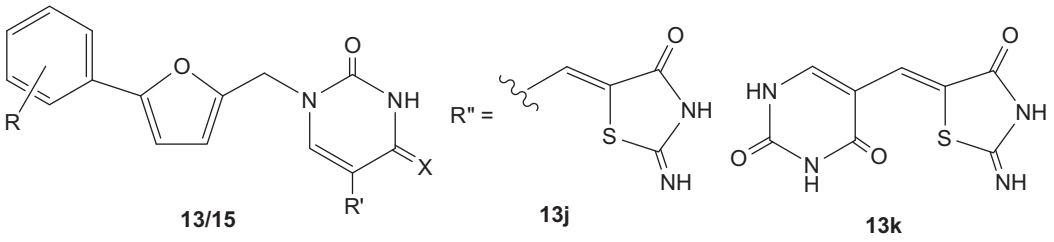


Scheme 2. Synthesis of compounds **13**.



Scheme 3. Synthesis of compounds **15**.

Table 3
The evaluation of readthrough activity of compounds **13** and **15**



Entry	Compd	R	R′	X	FC-ATM (Delta-FI, 30 μM)	IRIF-ATM (%) (30 μM)
1	**13a**	2-NO_2	H	O	4.94	10.67
2	**13b**	2-NO_2	F	O	2.08	14.5
3	**13c**	2-NO_2	Me	O	Negative	Negative
4	**13d**	2-NO_2	CHO	O	2.24	16.0
5	**13e**	H	H	O	3.42	9.4
6	**13f**	H	Me	O	3.80	22.75
7	**13g**	2-F	H	O	5.43	20
8	**13h**	2-F	Me	O	6.16	9.6
9	**13i**	3-Cl	H	O	5.07	11.33
10	**13j**	2-NO_2	R″	O	2.42	18
11	**13k**				5.32	17
12	**15a**	2-NO_2	H	NH	Negative	Negative
13	**15b**	2-NO_2	F	NH	Negative	Negative

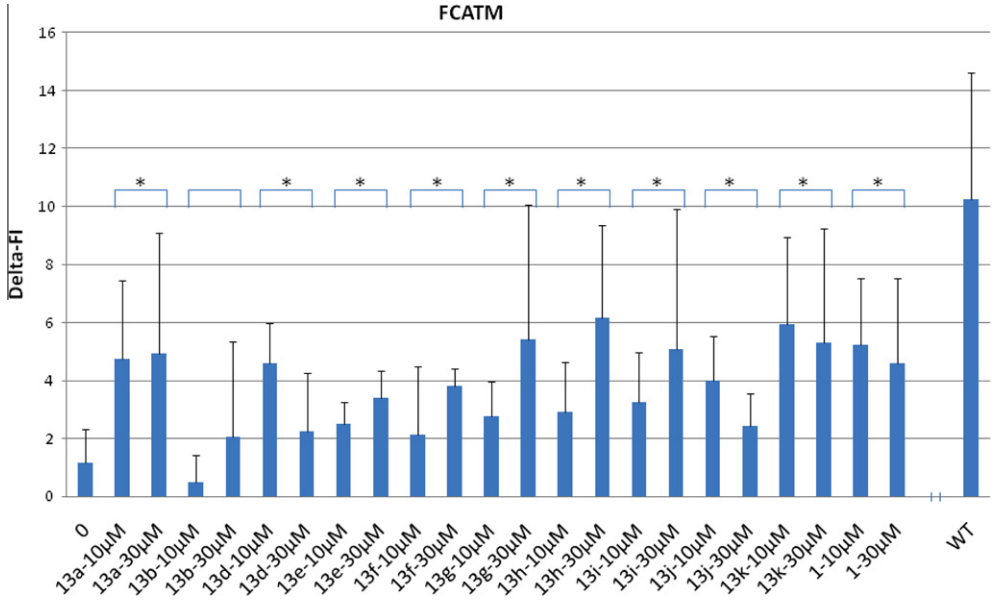


Figure 5. Measurement of readthrough activity of compounds **13** using FC-ATMs1981 in A-T cells. After 4 days of treatment with a compound, A-T cells were irradiated for 10 Gy and collected for assaying. Wild type cells (WT) were used as a positive assay control. The data from 3 sets of independent experiments are summarized here. The increased Delta-FI (difference of fluorescent intensity before and after IR) indicated the restoration of ATM autophosphorylation of at Serine 1981. All RTC-treated cell samples showed significant difference as compared to untreated cells (0), except **13b** (*p <0.05, by t-tests comparing paired concentrations of treated to untreated cells).

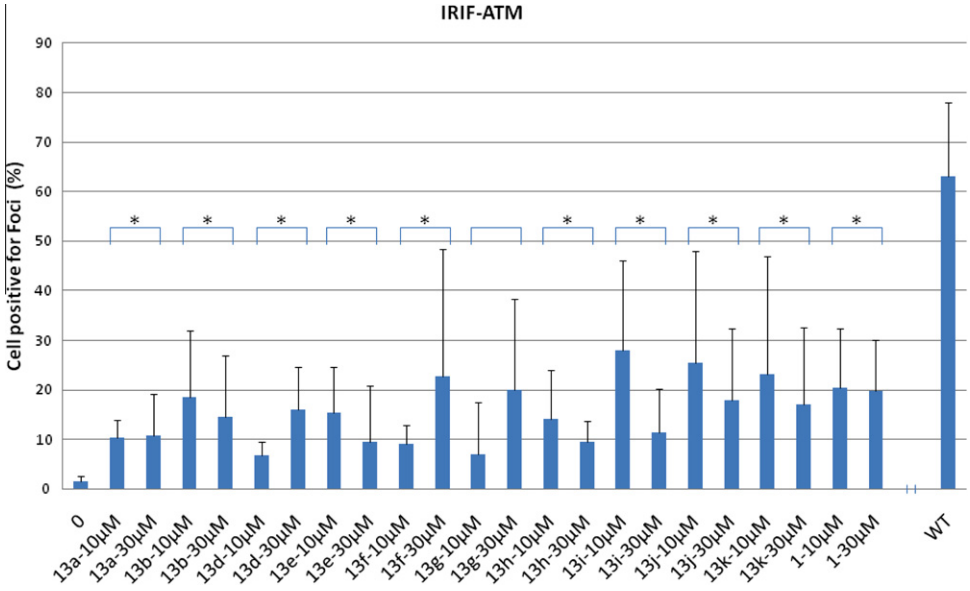


Figure 6. Measurement of readthrough activity of compounds **13a** using an ATMs1981 nuclear foci formation assay (IRIF-ATM) in A-T cells. After 4 days of treatment with a compound, A-T cells were irradiated for 10 Gy and collected for assaying. Wild type cells (WT) were used as a positive assay control. The data from >3 sets of independent of experiments are summarized here. All RTC-treated cell samples showed significant differences as compared to untreated cells (0), except **13g** ($^{*}p$ <0.05, by *t*-tests comparing paired concentrations of treated to untreated cells).

hydrogen bonding (Fig. 4). Consequently, we decided to substitute these thiazolidinone units with various pyrimidine bases, **F**, for example, uracil, 5-fluoro- and 5-methyluracil (thymine), and cytosine units. The synthesis of these novel analogs was relatively easy and involved hydride reduction of the 5-arylfurfurals **5** to give the alcohols **10** in good yields (Scheme 2). Conversion of the alcohols into the bromides **11** with PBr_3 followed by reaction of the crude bromides **11** with an excess (10 eq) of the desired pyrimidine bases **12a–d** gave good yields (57–88%) of the 1-pyrimidinylmethyl furans **13a–d**. An analogous series of reactions, beginning with other aldehydes **5**, permitted the formation of the pyrimidines **13e–i**. Condensation of the 2-imino thiazolidin-4-one **6a** with the aldehyde **13d** gave the analog **13j** having both a pyrimidinone and the thiazolidinone imine. Finally, condensation of the pyrimidinecarboxaldehyde with **6a** gave the novel bicyclic analog **13k** having just a pyrimidinone coupled to a thiazolidinone. The cytosine analogs were prepared by reaction of cytosine and 5-fluorocytosine **14a** and **b** with the nitro aldehyde **11** (R = 2-NO_2) in the presence of cesium carbonate in DMF to give **15a** and **b** in 67% and 69% yield, respectively (Scheme 3).

These two series, **13** and **15**, were again tested for readthrough activity using the assay in which the ATM protein kinase activity was measured by functional flow cytometry (FC-ATM) (Table 3 and Fig. 5). A second assay, irradiation (IR)-induced ATMs1981 nuclear foci formation (IRIF-ATM), was also used to confirm the readthrough activity of these compounds (Table 3 and Fig. 6). Table 3 summarizes the data for the analogs **13** and **15** (at 30 μM), in which the groups R, R′, and X were varied. More detailed data are reported in Figure 5 (for FC-ATM assay), and in Figure 6 (for IRIF-ATM assay). Interestingly, all of the pyrimidinedione analogs **13** (R′ = H, F, Me, and CHO), with the exception of **13c**, showed good activity while neither of the corresponding cytosine analogs, **15**, had any. Remarkably, the simple bicyclic analog **13k** showed good activity in both assays. Thus, the pyrimidinedione unit serves as a good structural replacement for the 2-imino and 2-thioxo thiazolidin-4-one units.

In summary, we have prepared a set of analogs of the HTS hit RTC13 **1** with variations on both the aryl substituent and the thiazolidinone rings **7** and **9** which show good readthrough activity. Moreover, we have designed and prepared novel compounds in which a pyrimidinedione unit replaces the thiazolidinone ring **13** and these also show excellent readthrough activity. Further work in this area is underway and will be reported in due course.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.07.107.

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