

# Translational readthrough-promoting drugs enhance pseudoknot-mediated suppression of the stop codon at the Moloney murine leukemia virus *gag-pol* junction

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Translational readthrough-promoting drugs enhance the incorporation of amino acids at stop codons and can thus bypass premature termination during protein synthesis. The polymerase (Pol) proteins of Moloney murine leukemia virus (MoMLV) are synthesized as a large Gag–Pol fusion protein, formed by the readthrough of a stop codon at the end of the *gag* ORF. The downstream *pol* ORF lacks its own start codon, and Pol protein synthesis is wholly dependent on translation of the upstream *gag* gene and the readthrough event for expression. Here, we explored the effects of readthrough-promoting drugs – aminoglycoside antibiotics and the small molecule ataluren – on the efficiency of readthrough of the stop codon in the context of the MoMLV genome. We showed that these compounds increased readthrough of the stop codon at the MoMLV *gag-pol* junction *in vivo* above the already high basal level and that the resulting elevated *gag-pol* readthrough had deleterious effects on virus replication. We also showed that readthrough efficiency could be driven to even higher levels *in vitro*, and that the combination of the small molecules and the RNA structure at the MoMLV stop codon could achieve extremely high readthrough efficiencies.

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## INTRODUCTION

Retroviruses rely on recoding – alternative interpretations of mRNA during translation – to overcome termination at a stop codon separating two ORFs and thereby control production of viral proteins. The retroviral *pol* gene, encoding enzymes for virus replication, lies directly downstream of the *gag* gene, encoding viral structural proteins. The *gag* ORF ends with a stop codon, and during conventional reading of the viral mRNA by the translational machinery, only the Gag polyprotein precursor is produced. The Pol proteins are synthesized only when the stop codon is misread and translation continues into the *pol* reading frame to produce a large Gag–Pol fusion protein (Murphy & Arlinghaus, 1978; Murphy *et al.*, 1978; Oppermann *et al.*, 1977). The recoding event can be mediated by one of two mechanisms in different viruses. Many retroviruses, such as avian retroviruses (Jacks & Varmus, 1985), human immunodeficiency virus type 1 (HIV-1) (Wilson *et al.*, 1988), human T-cell leukemia

virus type 1 (HTLV-1) (Nam *et al.*, 1993) and HTLV-2 (Honigman *et al.*, 1995) and mouse mammary tumor virus (Moore *et al.*, 1987), and also some coronaviruses (e.g. severe acute respiratory syndrome coronavirus), utilize ribosomal frameshifting (reviewed by Plant & Dinman, 2008). A frameshift event readjusts the position of the ribosome on the mRNA to an alternative reading frame. The stop codon is thereby bypassed in the new frame, and translation continues into the downstream gene. In contrast, murine and feline leukaemia viruses use a readthrough event to synthesize the Gag–Pol precursor (Yoshinaka *et al.*, 1985a, b); the translating ribosome remains in the same reading frame, but the UAG *gag* stop codon is misread as a sense codon, and the amino acid glutamine is inserted at the point of normal termination (reviewed by Levin, 1993; Rein & Levin, 1992). Recoding in retroviruses, and in many other viruses, requires the presence of a secondary structure in the viral mRNA that forms downstream of the site of recoding (Du *et al.*, 1997; Panganiban, 1988; Parkin *et al.*, 1992;

Wills *et al.*, 1991). This structure is often a two-stem, two-loop pseudoknot that varies in sequence and length among viruses and is utilized to induce a frameshift in some retroviruses and a readthrough in others (Chamorro *et al.*, 1992; Chen *et al.*, 1995; Le *et al.*, 1991). The Moloney murine leukemia virus (MoMLV) pseudoknot has been studied extensively by mutational and biochemical means (Alam *et al.*, 1999; Felsenstein & Goff, 1992; Feng *et al.*, 1989a, 1992; Wills *et al.*, 1991). Recently, RNA structures of this pseudoknot have been determined by nuclear magnetic resonance, revealing alternative conformations that control the level of the structure's activity as a *cis*-acting readthrough-promoting factor (Houck-Loomis *et al.*, 2011).

Readthrough can also be induced on eukaryotic cellular mRNAs by aminoglycoside antibiotics and readthrough-promoting drugs (Burke & Mogg, 1985; Fan-Minogue & Bedwell, 2008; Manuvakhova *et al.*, 2000; Murphy *et al.*, 2006; Welch *et al.*, 2007). Aminoglycosides are naturally occurring amino-modified sugars, traditionally used as antibiotics, that interfere with translation by promoting miscoding during the elongation process through interactions with the rRNA of the decoding centre (Carter *et al.*, 2000; Fourmy *et al.*, 1996; Ogle *et al.*, 2001). The interaction mimics the rRNA conformations that allow incorporation of near and non-cognate amino acids, and can result in insertion of an amino acid at a stop codon (reviewed by Zaher & Green, 2009). Most aminoglycosides work preferentially on prokaryotic ribosomes due to subtle differences in rRNAs between kingdoms and to better uptake by prokaryotic cells (Hermann, 2005; Xie *et al.*, 2010), but at higher concentrations, aminoglycosides can promote miscoding in mammalian cells. This effect is exploited in treatment of diseases caused by alleles with premature termination codons such as Duchenne's muscular dystrophy, cystic fibrosis and cancer with truncated p53 expression (reviewed by Rowe & Clancy, 2009). Treatment with aminoglycosides induces readthrough at a frequency that allows enough translation of the full-length protein to restore normal function. At high enough doses, aminoglycosides cause gross defects in eukaryotic translation, allowing their use in selection for drug resistance in tissue culture (Southern & Berg, 1982). Nephrotoxicity and non-reversible ototoxicity are serious side effects from the administration of aminoglycosides, which limit their clinical use (reviewed by Karasawa & Steyger, 2011). Screens for small molecules able to promote readthrough with reduced toxicity, however, have revealed potential alternatives (Du *et al.*, 2008, 2009; Nudelman *et al.*, 2009; Welch *et al.*, 2007). One such compound, ataluren (formerly PTC124), has been shown to increase readthrough and has demonstrated therapeutic effects in clinical trials for treatment of muscular dystrophy and cystic fibrosis (Kerem *et al.*, 2008; Sermet-Gaudelus *et al.*, 2010; Welch *et al.*, 2007; Wilschanski *et al.*, 2011).

A specific ratio of Gag:Gag–Pol products is needed for proper virus replication, and thus the recoding event at the *gag–pol* junction of the murine leukaemia viruses is

tightly regulated to occur at a frequency of about 5 % during translation. An abnormally high production of Gag–Pol protein has been shown to have negative effects on assembly and maturation of infectious particles (Felsenstein & Goff, 1988; Shehu-Xhilaga *et al.*, 2001). Manipulating the Gag : Gag–Pol ratio downward by mutation has been shown to be strongly deleterious to virus replication, while increasing the ratio by separate expression of these proteins, by silencing expression of the termination factors eRF1 or -3, or with aminoglycosides, was well tolerated in one study (Csibra *et al.*, 2014).

Here, we examined the effects of readthrough-promoting drugs on eukaryotic recoding in the context of retroviral readthrough sequences. We investigated the ability of aminoglycosides and the small molecule ataluren to increase readthrough of the MoMLV *gag–pol* junction beyond the normal levels of recoding induced by the pseudoknot. We showed that pseudoknot-mediated readthrough can be increased by certain drugs both *in vivo* and *in vitro*, thereby demonstrating that the normal efficiency of readthrough induced by the pseudoknot is not a maximal limiting level. We also showed that an abnormal increase in readthrough can modestly inhibit virus replication.

## RESULTS

### Aminoglycosides increase readthrough of the stop codon at the MoMLV *gag–pol* junction

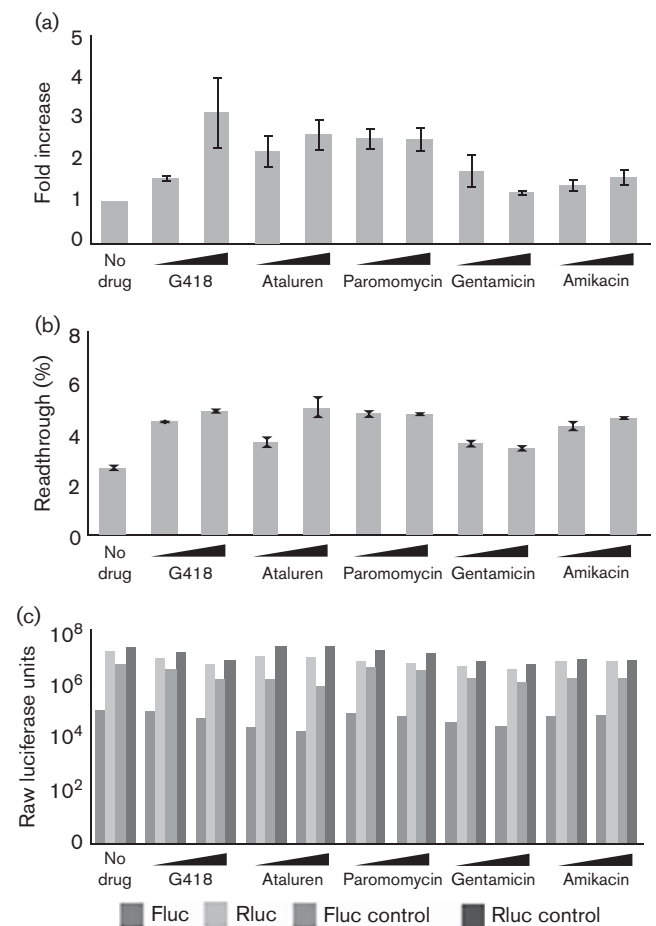
We tested the effect of several aminoglycosides and ataluren on readthrough of the stop codon at the MoMLV *gag–pol* junction in human 293A cells. We transfected cells with a dual-luciferase reporter DNA (MoMLV-PK) in which the MoMLV *gag–pol* junction region containing the *gag* stop codon (UAG), pseudoknot and relevant flanking nucleotides was placed downstream from the coding sequences for *Renilla* luciferase and upstream of the coding sequences for firefly luciferase (Green *et al.*, 2012). The efficiency of readthrough was then determined by measuring the ratio of firefly luciferase to *Renilla* luciferase activity. To normalize levels and to control for general effects on translation not related to readthrough, we performed parallel transfections of a control plasmid in which the UAG stop codon had been changed to CAG, directing maximal readthrough. The percentage readthrough was calculated as the ratio of firefly : *Renilla* luciferase in the MoMLV WT (containing the stop codon) context divided by the ratio of firefly : *Renilla* luciferase expressed by the no-stop control plasmid, multiplied by 100. Finally, the fold increase in these values upon addition of drug was determined in comparison with the value of the no-drug control, which was set to unity. We note that this readout of the efficiency of stop codon readthrough yields experimental values with a very tight distribution and very small errors across repeat assays (Houck-Loomis *et al.*, 2011; Green *et al.*, 2012).

We initially tested G418 at several concentrations in 293A cells and found that readthrough was consistently and significantly increased in a dose-dependent manner (Fig. 1a, b). We tested several other aminoglycosides: paromomycin (humetin), which is used to treat protozoan infections such as leishmaniasis (Banerjee *et al.*, 2011; Musa *et al.*, 2010) and cryptosporidiosis (reviewed by Cabada & White, 2010); gentamicin, a less toxic derivative of G418 that is widely used therapeutically; amikacin (amikin), used in the treatment of enterobacteria (Al-Tawfiq *et al.*, 2009) and some tuberculosis infections (Caminero *et al.*, 2010; Filippini *et al.*, 2010); and the non-aminoglycoside ataluren. G418 had the greatest effect on readthrough, with changes of more than threefold at the highest concentrations used (Fig. 1a). Ataluren and paromomycin increased the occurrence of readthrough over twofold. Gentamicin had a slight effect on increasing readthrough in some experiments, but the effects were not clearly dose dependent or consistently observed in all experiments. Amikacin treatment also resulted in only a slight increase in readthrough.

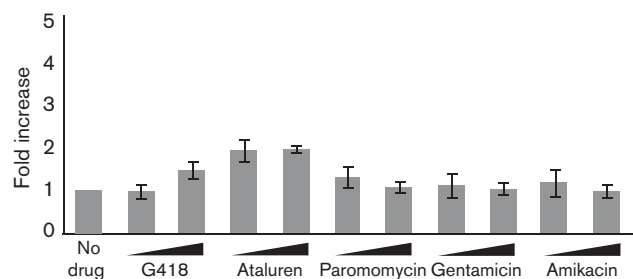
Ataluren has been reported to stabilize firefly luciferase and prevent its degradation, and so might cause higher signals by increasing the luciferase half-life rather than by effects on translation (Auld *et al.*, 2009, 2010). We observed no increase in firefly luciferase levels expressed by the control plasmid in ataluren-treated versus the no-drug control in our trials (Fig. 1c) and therefore concluded that increases in firefly luciferase signal in the presence of ataluren in our experimental system could be attributed to increased translation and not stabilization of the reporter protein.

G418 at higher concentrations had some global impact on translation, as all luciferase signals decreased slightly (Fig. 1c; compare Rluc and Rluc-control levels for G418 with the corresponding no-drug values). Gentamicin, although considered less toxic than G418, also decreased overall luciferase levels, with little effect on readthrough. Similarly, amikacin, which is well tolerated as a therapeutic antibiotic, also decreased overall translation. These decreases in luciferase signals may be due to a general inhibition of translation, or to an increase in misreading and production of non-functional luciferase proteins. Global effects on cell metabolism or viability caused by drug toxicity may also be a factor, particularly in the case of G418.

To verify that the increase in readthrough was directly attributable to the presence of the active forms of the readthrough-promoting drugs, we repeated the assays in neomycin (neo)-resistant 293T cells (Fig. 2). 293T cells contain the bacterial resistance gene *neo*, which encodes an aminoglycoside 3'-phosphotransferase, an enzyme that modifies the antibiotics and renders them inactive. All of the readthrough-promoting activities of the aminoglycosides were reduced in these cells. G418 demonstrated a significantly reduced ability to increase readthrough of the MoMLV *gag-pol* junction as expected, and paromomycin, gentamicin and amikacin did not show any increase in



**Fig. 1.** Effects of aminoglycosides and ataluren on readthrough at the MoMLV *gag-pol* junction. (a) Fold increase in readthrough at the MoMLV *gag-pol* junction in 293A cells induced by various small molecules, relative to the no-drug control. 293A cells were transfected with MoMLV-PK dual-luciferase reporter, and treated with increasing concentrations of aminoglycosides or ataluren as described in Methods. Readthrough efficiency was determined from the ratio of luciferase activities relative to the ratio of a control construct lacking the stop codon, and then expressed relative to the no-drug control, which was set to unity. The graph shows results from three trials per condition. Error bars represent SEM of mean fold changes in the various trials ( $n=3$ ). Drug concentrations were: G418, 0.25 and 0.5 mg ml<sup>-1</sup>; ataluren, 0.5 and 1  $\mu$ g ml<sup>-1</sup>; paromomycin, 0.5 and 1  $\mu$ g ml<sup>-1</sup>; gentamicin, 25 and 50  $\mu$ g ml<sup>-1</sup>; amikacin, 1.5 and 3 mg ml<sup>-1</sup>. (b) Absolute levels of readthrough efficiency in drug-treated 293A cells relative to the control construct lacking a stop codon. The graph shows a representative single experiment of 293A cells transfected with MoMLV-PK dual-luciferase reporter and treated with drugs. Error bars represent SD among triplicate luciferase readings in this experiment ( $n=3$ ). (c) Raw luciferase values in drug-treated 293A cells for Fluc and Rluc constructs with the stop codon, and for control constructs without the stop codon, as indicated, used to generate the data in (a) and (b). The graph shows the mean luciferase values of triplicate readings of each condition ( $n=3$ ).

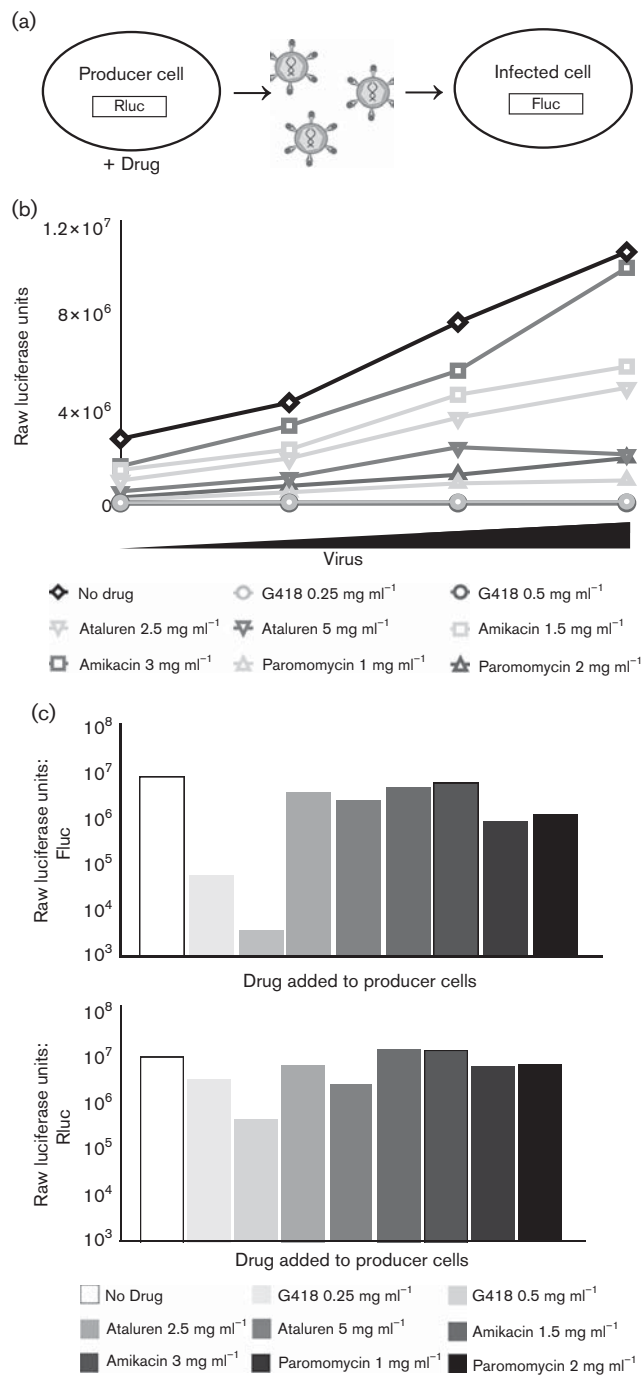


**Fig. 2.** Effects of aminoglycosides and ataluren on readthrough at the MoMLV *gag-pol* junction in neo-resistant 293T cells. 293T cells were transfected with the MoMLV-PK dual-luciferase reporter and treated with aminoglycosides or ataluren at two concentrations, as detailed in Fig. 1. The graph shows a representative experiment showing the fold increase of readthrough at the MoMLV *gag-pol* junction in neo-resistant 293T cells. Error bars represent SEM of average fold changes among triplicate readings in this experiment ( $n=3$ ).

readthrough in these cells. However, ataluren, which has a molecular structure unrelated to that of aminoglycosides and for which the mechanism of action is not precisely known, retained its readthrough-promoting activity. This result suggested that the increase in readthrough by the aminoglycosides is sensitive to neo-induced phosphorylation and that the mechanism of action of ataluren is distinct from that of the aminoglycosides.

### Aminoglycosides and ataluren decrease virus replication

We next examined the effect of the drug-induced increases in readthrough on the production of infectious virus particles. To measure virus production, we transfected 293A cells with a mixture of three DNAs: a plasmid expressing an MoMLV vector genome expressing the firefly luciferase gene, a plasmid expressing the vesicular stomatitis virus envelope glycoprotein (VSV-G) and a plasmid containing the complete MoMLV *gag-pol* genes. The cells were then treated with readthrough-promoting drugs to alter the Gag:Gag-Pol ratio. To test for effects of the drugs on transfection efficiency or on the health or viability of the cells, a fourth DNA, encoding *Renilla* luciferase, was included with the transfecting DNAs, and levels of *Renilla* luciferase were determined. Culture medium from the virus-producing cells was collected 48 h after transfection and serial dilutions of these virus harvests were used to infect naïve 293A cells. Expression of firefly luciferase in the infected-cell lysates was measured to gauge the amount of infectious virus produced by the drug-treated cells (Fig. 3a). We note that, although cells infected with reporter virus are exposed to low levels of residual drug in the medium, there is no recoding signal in the reporter vector, and firefly luciferase expression is independent of readthrough.



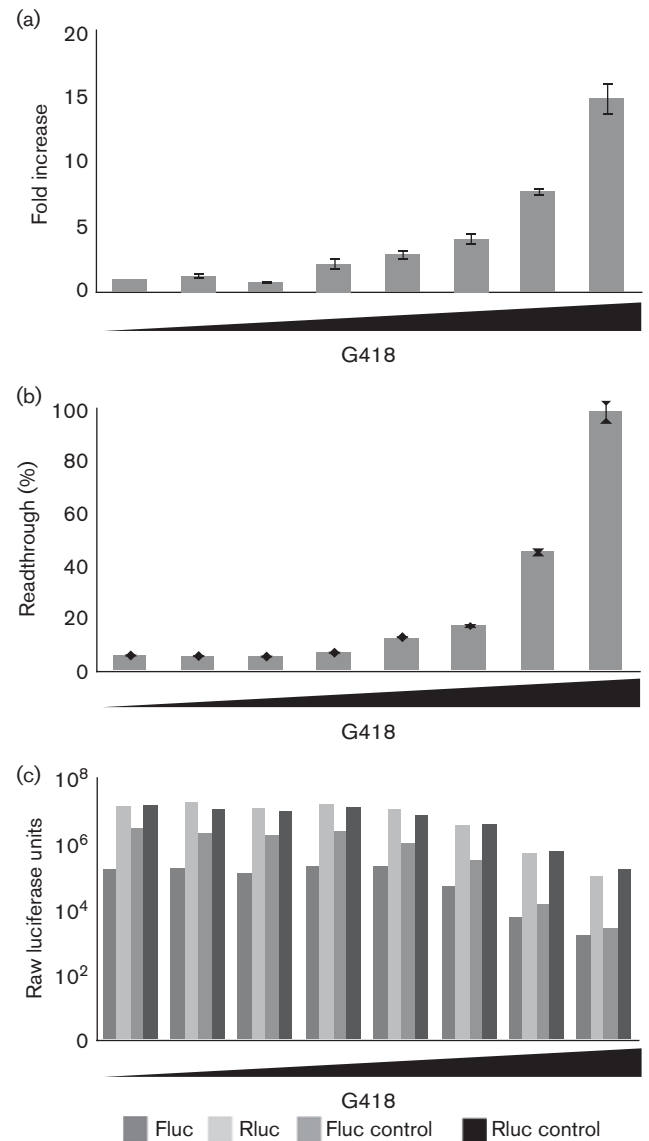
**Fig. 3.** Effects of aminoglycosides and ataluren on virus replication. (a) Schematic of the transducing virus assay. Producer cells were generated by transfection with plasmids expressing MoMLV *gag-pol* and VSV-G *env*, a viral vector carrying firefly luciferase as a reporter gene, and a DNA encoding *Renilla* luciferase. Producer cells were treated with readthrough-promoting drugs at the indicated concentrations, and virus was harvested from producer cells and used to infect naïve cells. Firefly luciferase activity was measured in infected cells as an indicator of production of virus. *Renilla* luciferase activity was measured in producer cells as an indicator of overall translational competence. (b) Firefly luciferase activity in infected cells, after infection with virus collected from

producer cells treated with the indicated drugs. Cells were infected with increasing concentrations of virus collected from drug-treated cells (serial dilutions of 1:16, 1:8, 1:4, 1:2, titres increasing from left to right). Values are the means of duplicate assays ( $n = 2$ ). (c) Example of infections with the 1:8 virus dilution used in (b). Upper panel: virus production as measured by expression of firefly luciferase in infected cells. Lower panel: translational competence of producer cells treated with drugs as measured by *Renilla* luciferase activity.

Four compounds – G418, ataluren, amikacin and paromomycin – were tested for their effects on virus production. Cells treated with G418 produced very little virus (Fig. 3b), with a reduction of over 4 logs in transduced levels of firefly luciferase compared with virus produced in untreated cells (Fig. 3c). However, *Renilla* luciferase expression in the transfected producer cells was also profoundly decreased, suggesting that the effect on virus was probably attributable to general drug toxicity on the transfected cells. Paromomycin treatment had the next most significant effect on production of virus, with transduction of firefly luciferase into infected cells reduced by approximately 10-fold. *Renilla* luciferase expression in the drug-treated producer cells was only slightly reduced from the no-drug control, suggesting very limited toxicity. Ataluren treatment caused only a very small decrease in virus production and transduction of firefly luciferase activity in infected cells, and minimal effects on *Renilla* levels in the transfected cells. Interestingly, despite amikacin's modest but significant ability to increase readthrough of the MoMLV *gag-pol* junction dual-luciferase reporter, the drug showed a negligible effect on virus production and no change, or even a modest increase, in *Renilla* signal in the producer cells.

### Aminoglycosides increase MoMLV readthrough *in vitro*

To assess the effects of the readthrough-promoting drugs without the limitations of drug uptake and toxicity in live cells, we performed coupled *in vitro* transcription and translation (IVT) reactions that expressed the MoMLV *gag-pol* junction dual-luciferase reporter. We prepared IVT reactions with reporter constructs and increasing concentrations of aminoglycosides, and measured luciferase levels generated during the reactions as before. We tested G418, paromomycin and ataluren because of their enhancement of readthrough *in vivo*. G418 caused a dramatic enhancement of MoMLV readthrough in a dose-dependent manner (Fig. 4a, b). G418 had little effect on readthrough efficiency at the lowest concentrations tested (5 and 20 ng ml<sup>-1</sup>), and a small increase in readthrough with no global effect on translation, compared with the no-drug control, at moderate concentrations. Readthrough was increased twofold at 300 ng ml<sup>-1</sup> but with some reduction in control reporter luciferase



**Fig. 4.** Effects of G418 on readthrough of the MoMLV *gag-pol* junction *in vitro*. (a) Mean fold increase above the no-drug control of readthrough of the MoMLV *gag-pol* junction during *in vitro* translation reaction with increasing concentrations of G418 [final concentrations shown are 0 (no drug), 5 ng ml<sup>-1</sup>, 20 ng ml<sup>-1</sup>, 80 ng ml<sup>-1</sup>, 300 ng ml<sup>-1</sup>, 1.25 μg ml<sup>-1</sup>, 5 μg ml<sup>-1</sup> and 20 μg ml<sup>-1</sup>], with means calculated from three trials at each concentration ( $n=3$ ). Error bars represent SEM of average fold changes in the various trials. (b) Readthrough levels of the MoMLV *gag-pol* junction during *in vitro* translation reaction with increasing concentrations of G418; values are means of triplicate experiments ( $n=3$ ). Error bars represent SEM of average fold changes in the various trials. (c) Raw Fluc and Rluc luciferase values in IVT reactions performed with construct with a stop codon, and for control construct without a stop codon, as indicated, used to generate the data in panels (a) and (b). The graph shows mean luciferase values of triplicate readings of each condition ( $n=3$ ).



levels (Fig. 4c). This trend of increased readthrough with decreased control luciferase expression continued with consecutively higher G418 levels. The highest final concentration of  $20 \mu\text{g ml}^{-1}$  induced very high levels of readthrough, but the overall translation efficiency was profoundly impaired at this drug level. We concluded that, although G418 increases readthrough in a dose-dependent manner *in vitro*, it also negatively affects translation of all mRNAs.

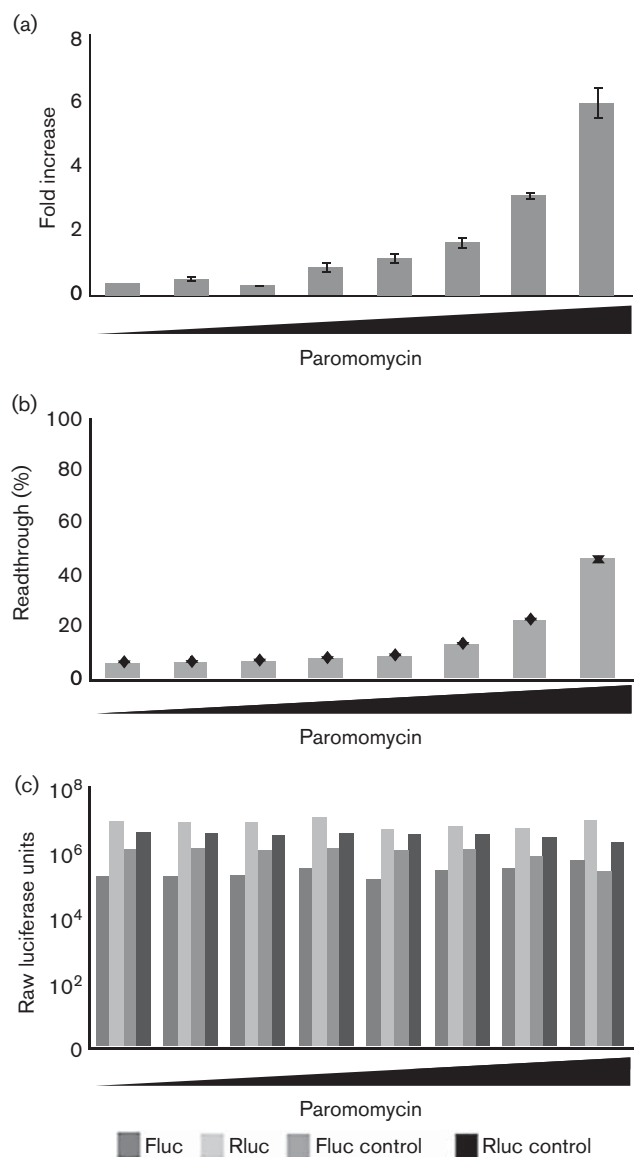
Paromomycin also caused a dose-dependent increase in readthrough of the MoMLV *gag-pol* reporter *in vitro*. As with G418, the readthrough efficiencies attained *in vitro* were considerably higher than the maximum measured *in vivo* in 293A cells, with the highest levels of paromomycin inducing a sixfold increase (Fig. 5a), which corresponded to over 40% readthrough efficiency (Fig. 5b). In contrast to the findings with G418, the raw levels of luciferase remained relatively stable, even at high concentrations of paromomycin, indicating that general translation was not impaired (Fig. 5c).

We also tested ataluren for effects on readthrough *in vitro* but were unable to observe any significant increases in readthrough (data not shown). These results suggested that ataluren may act indirectly *in vivo* or require cellular components that are missing from the *in vitro* reactions for its activity.

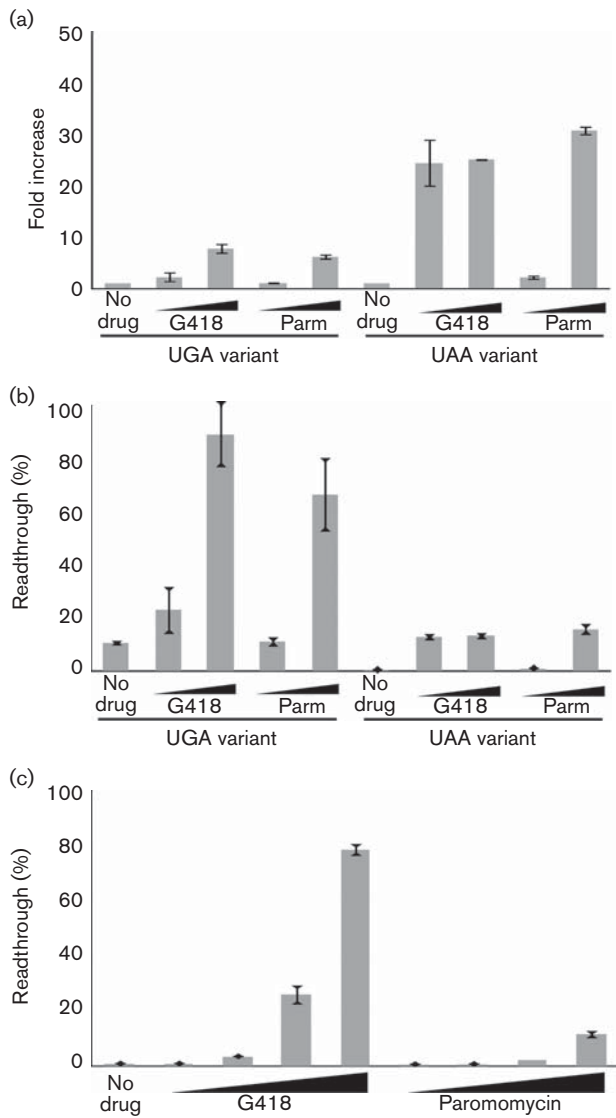
### Aminoglycosides increase readthrough of all three stop codons, of a hypoactive pseudoknot mutant and without viral pseudoknot sequences

We next examined the effects of G418 and paromomycin on a number of sequence variants of the MoMLV *gag-pol* junction. We tested luciferase constructs in which the WT UAG terminator was replaced by UGA and UAA stop codons. Viruses carrying these alternative stop codons are known to express Gag-Pol, with the insertion of distinct amino acids in the readthrough protein (Feng *et al.*, 1989b, 1990), and are at least partially replication competent (Jones *et al.*, 1989; Odawara *et al.*, 1991). G418 and paromomycin both increased readthrough with both alternative stop codons (Fig. 6a, b). Readthrough of UGA had a high basal level without drug, and was increased by the drugs to a very high absolute level, with a maximal increase of about fivefold. UGA has been observed to have a higher incidence of basal readthrough in non-viral mRNAs and appears to be more sensitive to drug-induced enhancement of readthrough than UAA. Readthrough of UAA, which is resistant to readthrough under conditions of normal translation (Manuvakhova *et al.*, 2000), had a low basal level and increased with both drugs, with a high fold increase of about 20-fold. These results indicated that, in the context of the MoMLV *gag-pol* junction, the same stop codon drug effects were observed as in studies of non-pseudoknot drug-mediated readthrough events.

We additionally tested the effects of G418 and paromomycin on a pseudoknot mutant with impaired readthrough-promoting ability. The G11C pseudoknot mutant contains a

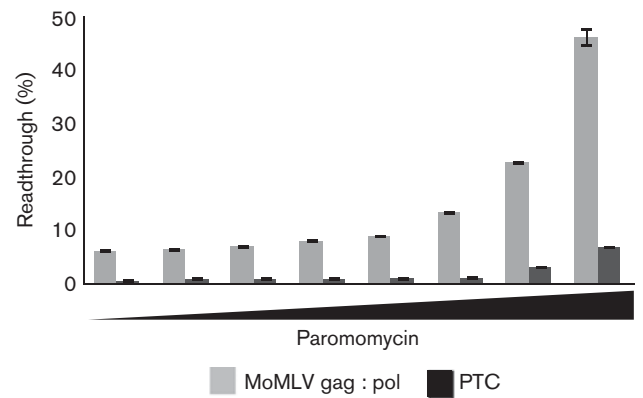


**Fig. 5.** Effects of paromomycin on readthrough of the MoMLV *gag-pol* junction *in vitro*. (a) Mean fold increase in readthrough of the MoMLV *gag-pol* junction during *in vitro* translation reactions with increasing concentrations of paromomycin [final concentrations: 0 (no drug),  $5 \text{ ng ml}^{-1}$ ,  $20 \text{ ng ml}^{-1}$ ,  $80 \text{ ng ml}^{-1}$ ,  $300 \text{ ng ml}^{-1}$ ,  $1.25 \mu\text{g ml}^{-1}$ ,  $5 \mu\text{g ml}^{-1}$  and  $20 \mu\text{g ml}^{-1}$ ]; means were calculated from at least three trials per concentration. Error bars represent standard error of average fold changes in the various trials. (b) Readthrough levels of MoMLV *gag-pol* junction during *in vitro* translation reactions with increasing concentrations of paromomycin; representative trial shown; values are means of triplicate experiments ( $n=3$ ). Error bars represent SEM of average fold changes in the various trials. (c) Raw Fluc and Rluc luciferase values in IVT reactions performed with construct with a stop codon, and for control construct without a stop codon, as indicated, used to generate the data in (a) and (b). The graph shows mean luciferase values of triplicate readings of each condition ( $n=3$ ).



**Fig. 6.** Effects of G418 and paromomycin on MoMLV *gag-pol* junction mutants. (a) Mean fold increase in readthrough frequencies of MoMLV *gag-pol* junction variants with UGA (left) and UAA (right) stop codons replacing the WT UAG stop codon during *in vitro* translation reactions containing two concentrations of G418 (80 and 300 ng ml<sup>-1</sup>) or paromomycin (Parm; 1.5 and 5 µg ml<sup>-1</sup>), expressed relative to the no-drug control, which was set to unity. Error bars represent SEM of duplicate luciferase readings ( $n=2$ ). (b) Readthrough frequencies used in generating graph shown in (a). Error bars represent SEM of duplicate luciferase readings ( $n=2$ ). (c) Readthrough frequencies of MoMLV *gag-pol* hypoactive pseudoknot mutant G11C during *in vitro* translation reactions with increasing concentrations of G418 or paromomycin (5 ng ml<sup>-1</sup>, 80 ng ml<sup>-1</sup>, 1.25 µg ml<sup>-1</sup> and 20 µg ml<sup>-1</sup>). Error bars represent SEM of triplicate luciferase readings ( $n=3$ ).

change of the 11<sup>th</sup> nt after the *gag* stop codon, which disrupts base pairing in the first stem of the pseudoknot structure. Viruses with this mutation do not replicate (Felsenstein & Goff, 1992), probably due to impaired readthrough. The



**Fig. 7.** Effects of paromomycin on readthrough of a no-pseudoknot PTC and WT MoMLV *gag-pol* sequence. The readthrough frequency for a dual-luciferase reporter with a no-pseudoknot PTC and for the WT MoMLV reporter were determined in the presence of increasing concentrations of paromomycin (no drug; 5 ng ml<sup>-1</sup>, 20 ng ml<sup>-1</sup>, 80 ng ml<sup>-1</sup>, 300 ng ml<sup>-1</sup>, 1.25 µg ml<sup>-1</sup>, 5 µg ml<sup>-1</sup> and 20 µg ml<sup>-1</sup>). Error bars indicate SD of means from representative experiments with each reporter ( $n=3$ ).

hypoactive G11C luciferase reporter showed enhanced readthrough in IVT reactions supplemented with increasing amounts of G418 (Fig. 6c). Paromomycin was also able to increase readthrough of this reporter, albeit with more modest fold changes. Notably, both drugs increased the readthrough of the hypoactive pseudoknot mutant to a lower level than they did with the WT pseudoknot. These results indicated that deficits in the readthrough-promoting activity of the G11C mutant pseudoknot are not fully overcome by the action of readthrough-promoting drugs.

To determine whether the paromomycin induced a specific or distinctive enhancement of readthrough in the presence of the pseudoknot structure, we directly compared the MoMLV pseudoknot and a no-pseudoknot reporter in the IVT system. The experimental premature termination codon (PTC) reporter contained the same UAG-G stop codon sequence as our MoMLV *gag-pol* junction dual-luciferase reporter, but the pseudoknot sequence was replaced with a portion of the collagen ORF, which is not known to contain any secondary structure; a matched control plasmid contained a CAG-G sequence to allow maximum expression of the luciferases to use as normalization. Both PTC and MoMLV reporters showed an increase in readthrough with paromomycin. The basal readthrough level of the PTC reporter was very low (0.4%), and increased 16-fold to 6.8% with the highest concentration of paromomycin (Fig. 7). This was a greater fold increase in readthrough for the PTC reporter than for the MoMLV *gag-pol* junction reporter, although the maximal readthrough frequency of the MoMLV reporter was much higher at almost 50%. This result suggests that paromomycin is highly active even without the pseudoknot.

## DISCUSSION

We examined the effect of aminoglycoside antibiotics and the small molecule ataluren on the readthrough efficiency of the MoMLV *gag-pol* junction. We showed that exposure of mammalian cells to specific aminoglycosides resulted in a dose-dependent increase in the already high basal readthrough levels at the MoMLV junction as measured by our dual-luciferase reporter system. The various drugs demonstrated distinctive behaviours. G418 and paromomycin exhibited the greatest effects on readthrough *in vivo*, while amikacin and gentamicin demonstrated little effect on MoMLV readthrough in this setting. Exposure of cells to ataluren also increased readthrough of the MoMLV *gag-pol* reporter. It has been reported that ataluren can interact reversibly with firefly luciferase to stabilize it and prevent its degradation; thus, high levels of readthrough can be mimicked by an accumulation of the reporter over the course of the assay (Auld *et al.*, 2009, 2010), but this process did not seem to be occurring in our experimental conditions. We also showed that the aminoglycoside effects on readthrough were not observed in 293T 'neo'-resistant cells. Therefore, unsurprisingly, inactivation of the drugs by modifying enzymes in drug-resistant cells prevented their ability to enhance readthrough.

The detailed mechanisms of action of the aminoglycosides on readthrough are not clear. Most likely, they involve interactions with the ribosomal A site of the elongating ribosome, subtly altering the function of the decoding machinery and affecting the ratio of binding of the termination factors versus tRNA at the time of recognition of the stop codon. The increase in readthrough probably does not involve interactions of the drug with secondary structures in the viral mRNA, such as the pseudoknot itself, although this possibility cannot be strictly ruled out. The aminoglycosides do interact specifically with at least one viral RNA, the RNA dimerization elements of the HIV-1 genome (Ennifar *et al.*, 2003), and can prevent structural rearrangements required for the complete dimerization of the two copies of the HIV RNA genome during virion assembly (Bernacchi *et al.*, 2007; Ennifar *et al.*, 2006). This structure has no known role in recoding, however, and the MoMLV pseudoknot has no obvious structural similarity to the HIV-1 RNA dimerization region or to the ribosomal A site.

We showed that exposure of virus-producing cells to readthrough-promoting drugs can affect virus production. The titre of reporter virus was reduced in all cells infected with virus produced in the presence of drugs. Virus from G418-treated cells was reduced from 2 to 4 logs compared with the no-drug control. However, G418 was clearly toxic: *Renilla* luciferase levels in producer cells exposed to G418 were reduced in all trials, and a viability assay of G418-treated cells confirmed that viability was reduced as G418 concentration and readthrough efficiency increased (data not shown). Therefore, we were unable to determine whether it was the readthrough-promoting action of G418 or its general toxicity to the producer cells that was responsible for the decreased virus yield. Paromomycin

had the greatest effect on virus replication with the least disruption of translation in producer cells (measured by *Renilla* luciferase expression), with up to a 10-fold reduction in virus yield, suggesting that this compound is the most promising lead for an effective antiviral drug. The structural differences responsible for the differential toxicity are not clear. Both G418 and paromomycin have an OH at the 6' position of ring I, which may be responsible for facilitating an interaction with the 18S rRNA that favours readthrough. However, the structure of an additional sugar (ring III) is different between the two, and is attached at a different position on ring II. The combination of ring III structure and linkage may contribute to the toxicity of G418 and/or higher tolerance of cells for paromomycin.

We also examined the effects of G418 and paromomycin on readthrough in an *in vitro* translation system. We found that G418 promoted high levels of readthrough but that the increases were associated with overall translational inhibition, consistent with the toxicity *in vivo*. Paromomycin caused moderate increases in readthrough frequency in a dose-dependent manner with less negative impact on overall translation. G418 and paromomycin also stimulated readthrough of alternative stop codons in the MoMLV *gag-pol* context, and of a hypoactive pseudoknot mutant. We were surprised to observe that ataluren showed no effects in our *in vitro* reactions. Its activity may require components in cells but missing from the *in vitro* reactions, or present at concentrations too low to contribute. The readouts used here could in principle provide an assay for the identification of factors that might recreate the ataluren effects seen *in vivo*.

A major conclusion from these experiments is that readthrough promoted by the MoMLV pseudoknot can be pushed beyond normal levels upon exposure to certain aminoglycosides. Thus, the results showed that the mechanism by which the MoMLV pseudoknot stimulates readthrough does not normally operate at maximal efficiency. These two stimulators of readthrough can act together and probably act independently. The results suggest that there may be more than one point of regulation that can be altered to promote miscoding. Finally, we note that our limited survey of aminoglycoside drug effects on MoMLV readthrough suggests that it may well be possible to usefully alter the rate of miscoding to reduce virus replication without cytotoxicity. The effects of paromomycin and ataluren are encouraging, indicating that similar drugs might have useful antiviral activity. Although any such compounds that act through this mechanism are not likely to be as potent as direct inhibitors of the viral reverse transcriptase, protease or integrase, the distinct target of their action would suggest that they would retain activity towards the more common drug-resistant viral mutants.

## METHODS

**Tissue culture.** 293A and 293T cells were grown in Dulbecco's Modified Eagle's Medium with 10 % FBS supplemented with L-glutamine plus 100 units/ml penicillin and 100 µg/ml streptomycin. Cells



were maintained in a 5 % CO<sub>2</sub> atmosphere at 37 °C, and were passaged at approximately 70 % confluence.

**Transfection and dosing of cell cultures.** Cells were plated at densities of 250 000 cells per well in six-well plates the day before transfection. Cells were transfected with 1 µg experimental or control reporter DNA using Fugene 6 Transfection reagent (Roche) at a ratio of 3 µl Fugene : 1 µg DNA; all transfections were adjusted with empty vector to contain the same total amount of DNA per well per experiment. At 2 h post-transfection, the medium was replaced and supplemented with aminoglycosides or ataluren. Drugs were used at the following final concentrations: G418, 0.25 and 0.5 mg ml<sup>-1</sup>; ataluren, 0.5 and 1 µg ml<sup>-1</sup>; paromomycin, 0.5 and 1.0 µg ml<sup>-1</sup>; amikacin, 1.5 and 3.0 mg ml<sup>-1</sup>; gentamicin, 25 and 50 µg ml<sup>-1</sup>. Cells were harvested for dual-luciferase analysis at 24 h post-transfection by lysis with 150 µl Promega Passive Lysis buffer per well.

**Dual-luciferase assay.** The dual-luciferase reporter system used here was essentially as originally described (Grentzmann *et al.*, 1998), and made use of MoMLV-based reporter DNA and assays utilized previously (Green *et al.*, 2012). Cell lysates from transfections (20 µl from each well) were transferred to opaque 96-well plates in triplicate. Dual-luciferase assays were performed using a Promega Dual Luciferase kit with buffers diluted 1 : 2 with dH<sub>2</sub>O. Assays were read on an Omega plate reader for a 24 s interval per well, with readings taken every half second for a total of 48 readings. One hundred microlitres of firefly luciferase reagent was dispensed for the first 12 s. Firefly signal was quenched by *Renilla* luciferase reagent addition at 12.5 s. Values from each interval of firefly (fluc) and *Renilla* (rluc) luciferase were summed in the Omega Mars Data Analysis program and exported to a spreadsheet program (Numbers) for analysis. The percentage recoding was calculated as [(fluc WT/rluc WT) × (rluc control/fluc control)] × 100, with WT representing signals from expression of plasmids containing the native recoding sequence and control representing signals from plasmids designed to eliminate recoding elements to allow maximal expression of both luciferases.

**Transducing virus assay.** 293A cells were plated at 250 000 cells per well in six-well plates and transfected the next day with 0.5 µg of a mix of three plasmids encoding components of a recombinant luciferase reporter virus [MoMLV *gag-pol* (pCMV-intron), firefly luciferase with the MoMLV packaging signal (pFbluc) and VSV-G envelope (pMDG)], along with DNA encoding *Renilla* luciferase and the human thymidine kinase reporter (pHRLTK) as a measure of translational health in the producer cell. At 2 h post-transfection, the medium was replaced and supplemented with readthrough-promoting drugs. At 24 h post-transfection, virus-containing supernatants were collected from producer cells and used to infect naive 293A cells at 1 : 2, 1 : 4, 1 : 8 and 1 : 16 dilutions. Producer cells were assayed for *Renilla* luciferase activity after virus collection. Infected cells were assayed for firefly luciferase activity at 24 h post-infection.

**Assay for enhanced readthrough during translation *in vitro*.** Drug effects on readthrough occurring during *in vitro* translation reactions were determined by assaying expression of dual-luciferase reporter plasmids using Promega T7 TnT (Transcription and Translation) reagents. Reactions were assembled on ice with the following components: 6.25 µl TnT lysate, 0.5 µl TnT reaction buffer, 0.25 µl T7 polymerase, 0.25 complete amino acid mix, 0.25 µl reporter plasmid at 1 µg µl<sup>-1</sup>, 0.5 µl drug solution and 4.5 µl nuclease-free H<sub>2</sub>O. Reactions were incubated for 2 h at 30 °C. Reaction mixes were diluted with passive lysis buffer, and assayed for *Renilla* and firefly luciferase activity as described above for cell lysates.

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