Ammonium and arsenic trioxide are potent facilitators of oligonucleotide function when delivered by gymnosis

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ABSTRACT
Oligonucleotide (ON) concentrations employed for therapeutic applications vary widely, but in general are high enough to raise significant concerns for off target effects and cellular toxicity. However, lowering ON concentrations reduces the chances of a therapeutic response, since typically relatively small amounts of ON are taken up by targeted cells in tissue culture. It is therefore imperative to identify new strategies to improve the concentration dependence of ON function. In this work, we have identified ammonium ion (NH₄⁺) as a non-toxic potent enhancer of ON activity in the nucleus and cytoplasm following delivery by gymnosis. NH₄⁺ is a metabolite that has been extensively employed as diuretic, expectorant, for the treatment of renal calculi and in a variety of other diseases. Enhancement of function can be found in attached and suspension cells, including in difficult-to-transfect Jurkat T and CEM T cells. We have also demonstrated that NH₄⁺ can synergistically interact with arsenic trioxide (arsenite) to further promote ON function without producing any apparent increased cellular toxicity. These small, inexpensive, widely distributed molecules could be useful not only in laboratory experiments but potentially in therapeutic ON-based combinatorial strategy for clinical applications.

INTRODUCTION
The recent FDA approval for the marketing of eteplirsen, a phosphoromorpholidate antisense oligonucleotide (ASO) (1,2), for the treatment of Duchenne’s muscular dystrophy, has propelled the clinical development of splice-switching oligonucleotides (SSOs) (3). At the same time, drisapersen, a phosphorothioate (PS) SSO, which like eteplirsen was designed to produce exon skipping in the dystrophin mRNA, did not fare as well, missing its primary endpoint (the 6-min walking test). Though the use of SSOs as therapeutic molecules is promising and has shown to be well tolerated, including after multiple intrathecal administrations as in the case of nusinersen (4), their potential, as for any therapeutic antisense ON, is hampered by substandard delivery to their targeted cells. Attempts to improve efficacy by escalating ON doses frequently leads to unacceptable toxicity. Chemical modifications, such as locked nucleic acid (LNA) have proven to increase ON efficacy in vivo, but the required concentrations for some therapeutic applications, depending on the ON sequence, may also produce toxicity (5,6).

The LNA modification was first synthesized approximately 20 years ago (7,8). PS ONs containing LNA moieties are not only highly resistant to nucleases, but each LNA can increase the Tm of an RNA/PS LNA ON duplex by up to 2–6°C per residue (8,9). PS LNA ONs are active SSOs, having been shown to induce exon skipping in vivo as well as in vitro, especially in the colon, small intestine and liver (10). However, for therapeutic applications it is critically important to develop strategies that take advantage of these characteristics at low ON doses so that the potential for off target effects are reduced.

The PS LNA SSO employed in this work (SSO-654) is a 16mer. The LNA moieties are interspersed in the ON chain. As mentioned above, this substitution promotes nuclease stability and increases the stability of the ON hybrid with the nuclear pre-mRNA. At the same time, LNA substitution blocks the induction of RNase H activity, which would cleave the pre-mRNA and terminate exon skipping. To monitor ON efficacy, we employed a splice-switching model in which HeLa cells have been engineered to express the enhanced green fluorescent protein (EGFP; HeLa EGFP-654) (11,12). In this model, a mutated β-globin intron, which creates a new 5′ splice site and activates an upstream cryptic 3′ splice site, has been inserted into the EGFP...
coding sequence to generate an additional exon. This exon prevents canonical splicing and EGFP translation. Treatment of these cells with a SSO targeted to the introduced 5′ splice sites causes exon skipping and the reconstitution of the EGFP correct reading frame (12).

Several years ago, we (13,14) discovered the general principle that PS LNA ONs and other highly stabilized ONs such as 2′F-arabinose nucleic acids (15) can enter cells in the absence of any transfection vehicles and can also silence gene expression. This process is called gymnosis from the Greek word for naked, and is different from the process of ‘free uptake’ which only refers to the absence of transfection reagents (16), and has never been associated with silencing of gene expression. The process of gymnosis in tissue culture more resembles in vivo ON uptake in saline formulations than does the process of transfection (13), and is often used for both ASO and SSO experiments.

The concentrations of ON in the media for an optimal gymnosis experiment are often in the 250 nM–5 μM range. However, while gymnotic delivery of ONs generally results in excellent ON function, it still can be associated with inherent potential toxicity and with sub-optimal in vivo delivery. Thus, our strategy aims to improve the activity of PS LNA ONs (referred from this point on as ONs) after gymnosis in order to improve the concentration dependence of ON function. Ideally, such a strategy would employ small molecules that are distributed widely, are inexpensive and non-toxic at the concentrations employed.

However, there are few such small molecules to hand. In 2013, a small molecule known as Retro-1, which reduces the toxicity of plant and bacterial compounds (17) emerged from a high throughput screen. Retro-1 enhances SSO activity both in attached and suspension cells, and in mdx mice (19).

In this work, we elected to study the effects of ammonium ion (NH₄⁺) on the activity of ONs because of our previous results (20), which highlighted the importance of endosomal maturation for ON activity and because it has previously been shown that NH₄⁺ affects the maturation and outcomes of late endosomes (21–23).

Here, we demonstrate that NH₄⁺ can facilitate SSO activity in the HeLa EGFP-654 model in vitro, in the absence of, or with very limited, toxicity. NH₄⁺ also improves in vitro ASO activity both in attached and suspension cells, including in Jurkat T and CEM T cells, where gene silencing has historically been difficult. Finally, we demonstrate that NH₄⁺ can interact synergistically with arsenic trioxide (As III, arsenite in solution) to significantly promote ON function in cells.

MATERIALS AND METHODS

Cell culture

HeLa EGFP-654, HCT 116 and SW480 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 2 mM l-glutamine. LNCaP cells and the Jurkat T and CEM T lymphocyte cell lines were maintained in RPMI 1640 medium supplemented with 10% FBS and 2 mM L-glutamine. Cultures of all cell lines were maintained at 37°C in a humidified 5% CO₂ incubator.

Reagents and antibodies

Ammonium chloride (NH₄Cl), arsenic trioxide (As₂O₃) and oleic acid (OA) were purchased from Sigma-Aldrich (Milwaukee, WI, USA); Ambroxol hydrochloride (Amb) and ciclohexylamine were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). An arsenite solution was prepared by dissolving As₂O₃ in minimal volumes of 1 N sodium hydroxide (NaOH). The arsenite solution (referred to in the manuscript as As III) was then diluted with phosphate buffered saline to a concentration of 10 mM as a stock solution.

For Amb and OA, the desired concentrations were obtained by dissolving Amb in DMSO and OA in ethanol. Other compound solutions were prepared as per the manufacturer’s recommendations. The AR (N-20, used at a 1:1000 dilution) and GFP (sc-9996; also used at a 1:1000 dilution). antibodies were purchased from Santa Cruz Biotechnology. The β-catenin antibody (#4270; 1:1000) was purchased from Cell Signaling Technology (Danvers, MA, USA) and the anti-α-tubulin antibody (1:3000) from Sigma-Aldrich; the anti-BCL2 antibody (clone 124; 1:500) was from Dako (Santa Clara, CA, USA).

Antisense oligonucleotides

The sequences of ONs used in this work are listed in Table 1. All are phosphorothioates, with DNA given in lower case letters and LNA modifications in uppercase letters. ‘m’ = 5-methylcytosine. ONs were delivered by gymnosis by directly adding them into the culture media (14).

Western blot

Cells were harvested by trypsin digestion and washed once with PBS. Cell pellets were lysed in cold RIPA buffer containing protease inhibitors. Cellular RIPA lysates were sonicated for 2 s and then rested on ice for 5 min. Cell debris was removed by centrifugation at 12,000 × g for 10 min at 4°C. Protein concentrations were determined using the Pierce® BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Aliquots of cell extracts containing 30–40 μg of protein were resolved by SDS-PAGE gel electrophoresis and then transferred to PVDF membranes. After treatment with the appropriate primary and secondary antibodies, enhanced chemiluminescence was performed. Protein signals on the blot were quantified with the ImageJ program and protein expression was normalized to control = 100%.

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Quantitative RT-PCR

RNA was extracted from cells using RNA-STAT 60 (AMS Biotechnology, Abingdon, UK) as recommended by the manufacturer. First-strand cDNA was synthesized with the Super-Script® III First-Strand Synthesis System Kit (Invitrogen, Carlsbad, CA, USA). PCR was performed with Power SYBR GREEN PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). The PCR primers used in this study were: AR (5′-CCTGGCTTCCGCAACTTAC AC-3′; 5′-GGACTTGTGCACTGGGTCCTCA-3′); β-actin (5′-AGAAAATCTGGCAACCACACC-3′; 5′-CCATCT TCTTGTCGAAGTCC-3′). PCR assays were performed under optimized conditions at 95°C × 15 s, 54°C × 30 s, 72°C × 30 s for 40 cycles followed by a melting curve analysis. The AR mRNA levels were normalized to the expression of β-actin mRNA, which served as the internal control.

Flow cytometry

Cells were trypsinized, collected by centrifugation at 800 × g for 5 min and re-suspended in PBS for flow cytometric analyses. Flow cytometry data (a minimum of 10,000 events) were collected by a CyAn Flow Cytometer (Beckman Coulter, Brea, CA, USA), and analyzed by the FlowJo program (Tree Star, Inc., Ashland, OR, USA) to determine fluorescence intensity versus cell number. Cell apoptosis assays were performed with the Annexin V-FITC Apoptosis Detection kit (Abcam, Cambridge, UK) following the manufacturer’s recommendations. In brief, 5 × 10^3 cells were collected by centrifuging at 800 × g for 5 min and re-suspended in 500 μl of binding buffer. Annexin V-FITC and propidium iodide (PI) were added to the cell suspensions, which were incubated at room temperature for 5 min prior to the flow cytometric assays. For the viability assays, cells were harvested, re-suspended in PBS containing 1 μg/ml DAPI (Molecular Probes, Eugene, OR, USA) and incubated for 5 min prior to the flow cytometric experiments.

Cell growth assays

Cell growth and proliferation assays were performed with CellTiter 96® AQueous One Solution (Promega, Madison, WI) and by staining with sulforhodamine B (SRB). For CellTiter 96® AQueous assays, Jurkat T and CEM T (circa 2 × 10^4 cells per well) were seeded into 96-well plates and treated as indicated for two days. HCT 116 cells (2 × 10^4) were first seeded into 96-well plates overnight, and then treated as indicated for two days. 20 μl CellTiter 96® AQueous reagent was added to each well and incubated at 37°C for 1–2 h, prior to recording the absorbance at 490 nm with a microplate reader. For cell proliferation assays with SRB staining, HeLa EGFP-654 cells (2 × 10^4 per well) were seeded into 48-well plates overnight and treated the following day as indicated in the text and figure legend. Cells were fixed by adding an equal volume of cold 10% trichloroacetic acid solution to the culture media. The tissue culture plates were then placed at 4°C overnight. The following day, cells were washed three additional times with Millipore water, air-dried overnight and stained with 0.06% SRB for 30 min at room temperature in the dark. Cell-bound SRB was then solubilized in 10 mM Tris buffer (pH 10) and its absorbance determined at 510 nm by a microplate reader.

Statistical analysis

Quantifying the interaction between the ON and ammonium and As III treatments was performed by a combination index (CI) plot using the Chou-Talalay method (24). The significance of the data was calculated by determining the mean ± standard deviation (S.D.). One way ANOVA was performed to determine and compare the means for the samples analyzed in each experiment. For the experiments performed to assess ON efficacy, the adjusted P-values were obtained by employing a post-hoc Dunnett’s test to compare each treatment group to a single control. The Dunnett’s test was selected to avoid overestimation of the ON efficacy differences among pairs (25). However, so as not to underestimate toxicity by increasing the stringency of the statistical calculations with the Dunnett’s test (25), toxicity assays (e.g. Figure 2A and B, Supplementary Figure S7), were analyzed with a two-sample Student’s t-test without the adjustment of multiple comparisons.

The statistical analyses were performed with the multcomp package in R software (http://www.r-project.org/). Comparisons were considered to be significant when the adjusted or non-adjusted P-values were <0.05. All P-values are two-sided unless otherwise noted.

RESULTS

Ammonium ion enhances therapeutic SSO function in cells

The HeLa EGFP-654 cell line (11,12), stably expresses an EGFP pre-mRNA whose coding sequence is interrupted by the insertion of a mutated β-globin intron. The point mutation in this intron creates a new 5′ splice site, which in turn
allows for an upstream cryptic 3′ splice site to be utilized. The result is in the inclusion of an intron fragment (now an exon) within the spliced EGFP mRNA. The binding of SSO-654 to the aberrant 5′ splice site causes the skipping of the internal exon and restores EGFP expression (Figure 1A). We used this cell model to see if we could improve ON efficacy. We have demonstrated that ammonium chloride (NH₄⁺) significantly enhanced the function of a SSO (SSO-654, Figure 1B–F). Exposure to 5 mM NH₄⁺ for 2 days increased SSO-654 function and EGFP expression in HeLa EGFP-654 cells (Figure 1B–F) a minimum of 5-fold compared to SSO-654 alone as demonstrated by Western analysis (Figure 1C) and flow cytometric analysis (Figure
NH₄⁺ does not affect cell viability. (A and B) HeLa EGFP-654 cells were treated with the indicated concentrations of NH₄⁺ (A), or with [1 μM] SSO-654 in the presence or absence of [5 mM] NH₄⁺ (B) for 2 days. Cell growth and proliferation were assayed by SRB staining. The growth and proliferation of treated cells were normalized to non-treated controls as 100%, indicated in the graph by a value of 1 (100% cell growth after 48 hr of culture, SRB staining). The combination of NH₄⁺ and SSO-654 did not increase growth inhibition compared with NH₄⁺ alone ($P = 0.45$). For the toxicity experiments, the $P$-values were not adjusted to avoid the likelihood that the increased stringency of the statistical calculations could mask any differences in the treatment toxicity among the individual groups. Data are represented as the mean ± SD, $n = 3$. **$P < 0.01$, Student’s $t$-test. (C) The viability of HeLa EGFP-654 cells treated with [1 μM] SSO-654 with or without [5 mM] NH₄⁺ for 2 days. Cells were enzymatically harvested and stained with DAPI prior to flow cytometric assay. The flow cytometric data were analyzed using the FlowJo program (Tree Star, Inc., Ashland, OR). The combination of NH₄⁺ and SSO-654 increased the percentage of EGFP positive cells (51.2% versus 14.5% for the SSO-654 alone) (lower right quadrants), but the percentage of DAPI-stained dead cells (upper left quadrants) were comparable to the single treatments or the control (3.87% versus 4.16% for the SSO-654 alone, or 4.55% for the non-treated control).

1D–F). EGFP expression did not increase after treating the cells with a combination of NH₄⁺ and a non-targeting control ON (Figure 1B, compare Con-ASO versus NH₄⁺/Con-ASO). At lower ([0.5] to [2 mM]) NH₄⁺ concentrations, SSO-654-induced EGFP protein expression increased >2-fold (Figure 1C, compare lane 2 to lanes 3–5 and figures e–f), while treatment with 10 mM NH₄⁺ increased the percentage of EGFP positive cells and EGFP protein expression nearly 7-fold compared to cells treated with the SSO-654 alone (Figure 1D–F).

Consistent with these results, other ammonium-containing compounds such as ammonium acetate and ammonium bicarbonate, as suspected, produced comparable effects on SSO-654-mediated EGFP expression (Supplementary Figure S1). Taken together, these data demonstrated that NH₄⁺, at a range of clinically relevant concentrations, significantly facilitated the ability of SSO-654 to promote EGFP expression in a dose-dependent manner in HeLa EGFP-654 cells.

Ammonium ion exposure does not affect HeLa EGFP-654 cell viability

NH₄⁺ can be inhibitory to the growth of and toxic for mammalian cell cultures (26,27). Therefore, we examined whether the exposure NH₄⁺ combined with a SSO ON could affect the growth, proliferation, and viability of HeLa EGFP-654 cells. As shown in Figure 2A and B, NH₄⁺ at the lower concentrations [1-2 mM] did not affect the growth of these cells; NH₄⁺ at [5 mM] inhibited cell growth by about 15%, consistent with a previously study (26). However, the combination of ON SSO-654 and NH₄⁺ did not increase NH₄⁺-mediated growth inhibition ($P = 0.45$, Figure 2B). In cell viability assays employing flow cytometry (Figure 2C), treatment with NH₄⁺ and SSO-654 increased the percentage of EGFP-positive cells by nearly fourfold (Figure 2C, compare the lower right quadrants of SSO-654 and NH₄⁺/SSO-654), but did not increase the percentage of dead cells, which were detected by DAPI labeling (upper left quadrants).
Ammonium ion also facilitates ASO ability to silence gene expression in cancer cells

We then investigated whether NH$_4^+$ could facilitate the activity of PS LNA gapmer ASOs (ASOs) when delivered to a variety of cells by gyronesis. LNCaP prostate cancer cells were treated with an ASO targeting either the androgen receptor (AR-ASO), or BCL-2 (BCL2-ASO) or β-catenin (β-Cat-ASO) mRNAs, with or without added NH$_4^+$. As shown in Figure 3A, the presence of NH$_4^+$ facilitated ASO-induced gene silencing in a target-specific manner. The combination of treatment with NH$_4^+$ [5 mM] and the AR-ASO [1 μM] for three days led to an 80% reduction in AR protein expression (lane 4 in Figure 3A). Treatment with the AR-ASO alone resulted in only a 40% AR reduction (lane 3 in Figure 3A). Furthermore, treatment with the identical concentrations of the BCL2-ASO and NH$_4^+$ led to a nearly completely depletion of BCL-2 protein expression (98% BCL2-ASO and NH$_4^+$ versus 70% with the BCL2-ASO alone; lane 6 versus lane 5 in Figure 3A). Similarly, treatment with [1 μM] β-Cat-ASO + [5 mM] NH$_4^+$ led to a decrease of 90% in β-catenin protein expression vs. a 30% diminution in cells not treated with NH$_4^+$ (lane 8 versus lane 7 in Figure 3A).

We then obtained a dose response for the AR-ASO in LNCaP cells and demonstrated that 0.25 μM AR-ASO when combined with NH$_4^+$ induced greater AR reduction than 1 μM AR-ASO alone (lane 4 versus lane 2 in Figure 3B). This result was confirmed by qRT-PCR analysis, which demonstrated that the NH$_4^+$ and AR-ASO combination significantly reduced AR mRNA expression (by about 40%, adjusted P-value < 0.003), compared with the AR-ASO alone (Figure 3C). Furthermore, this outcome could be recapitulated in other cell lines: We confirmed that NH$_4^+$ increased the efficacy of the β-Cat-ASO by ∼2-fold in SW480 colon cancer cell lines and nearly eliminated β-catenin gene expression in HCT 116 colon cancer cells (lane 4 versus lane 3 in Figure 3D and E). In no case did NH$_4^+$ alone have any effect on the expression of those genes.

Ammonium ion facilitates ASO-directed gene silencing in transfection-resistant leukemia cells

We then examined whether NH$_4^+$ could promote gyronesis in non-adherent leukemia cells. These cells are often resistant to liposome-based transfection, a delivery method widely employed for the manipulation of gene expression. We examined Jurkat cells, a T lymphocyte cell line, and confirmed that these cells were indeed resistant to gene silencing by Lipofectamine® 3000-mediated-transfection of the BCL2-ASO (Figure 4A and not shown). This resistance was found even at high ASO concentrations (50–100 nM; lane 2 and 3 in Figure 4A). Some silencing of the BCL-2 protein expression was obtained by delivering [1 μM] BCL2-ASO via gyronesis (compare lane 4 to lane 1, Figure 4A). However, when combined with [1 μM] BCL2-ASO, the presence of NH$_4^+$ facilitated BCL2-ASO-mediated BCL-2 silencing by 65% (compare lane 5 to lane 4 and lane 1, Figure 4A) and nearly completely depleted BCL-2 protein expression when combined with 2 μM BCL2-ASO (compare lane 7, lane 6 and lane 1, Figure 4A).

We further confirmed the effect of NH$_4^+$ on BCL2-ASO-mediated gene silencing activity in CEM T-lymphoblastoid cells. These cells, like Jurkat T cells, are well-studied and transfection-resistant. Nevertheless, NH$_4^+$ was still capable of augmenting the ASO silencing of BCL-2 gene expression (Figure 4B). The combination of NH$_4^+$ with [1 μM] BCL2-ASO resulted in about a 50% reduction of BCL-2 protein expression (Figure 4B, lane 4), compared with only a 5% reduction obtained using [1 μM] BCL2-ASO alone (lane 2). The combination of NH$_4^+$ with [2 μM] BCL2 silenced BCL-2 protein expression by ∼75% (lane 6), versus [2 μM] BCL2-ASO alone, which only yielded a 40% decrease (lane 5).

Ammonium ion simultaneously facilitates gene targeting of two ONs in cells

We then investigated whether NH$_4^+$ could simultaneously facilitate the silencing ability of two ONs in cells in culture. As shown in Figure 5A, the combined treatment with NH$_4^+$ and both the SSO-654 and BCL2-ASO increased SSO-654-mediated EGFP expression (EGFP, lane 8 versus lane 3), in addition to enhancing BCL-2 silencing in HeLa EGFP-654 cells (BCL2-2, lane 8 versus lane 4). The activity of the SSO-654 + BCL2-ASO + NH$_4^+$ was comparable to that of the SSO-654 + NH$_4^+$ or the BCL2-ASO + NH$_4^+$ treatments individually (compare lane 8 to lane 5 or 6 in Figure 5A). We then titrated the concentration of the SSO-654 (evaluated by Western blotting, Figure 5B) or the BCL2-ASO (evaluated by flow cytometry, Figure 5C) in the presence or absence of NH$_4^+$, as shown in Figure 5B, we found that high concentrations of the SSO-654 (5 μM) could only partially reduce the BCL2-ASO-directed gene silencing (compare lane 9 with lane 3), while the SSO-654 at lower concentrations [1–2 μM] fully maintained the activity of both (compare lane 5 and 7 with lane 3 in Figure 5B). In contrast, when the concentration of SSO-654 was kept constant [1 μM] while the concentration of the BCL-2 ASO was increased up to [5 μM] the resulting increase in expression of EGFP was diminished (compare SSO-654/BCL2-2/NH$_4^+$ and SSO-654/BCL2-5/NH$_4^+$ with SSO-654/NH$_4^+$, Figure 5C top, and bar 8 and 9 with bar 6, Figure 5C bottom; n = 3; adjusted P-values of <0.05 and <0.001, respectively). At lower concentrations (up to [1 μM]) the BCL2 ON did not affect SSO-654 function (compare SSO-654/BCL2 [1 μM] /NH$_4^+$ with SSO-654/NH$_4^+$, Figure 5C top and bar 7 with bar 6, Figure 5C bottom). Consistently, other ASOs, including the AR-ASO, an ASO targeted to HER3 (HER3-ASO), and a non-targeting control ON (Con-ASO) all at [1 μM], when combined with NH$_4^+$ [5 mM] demonstrated only slight or no inhibition of NH$_4^+$ + SSO-654-mediated EGFP expression in HeLa EGFP-654 cells under the conditions of the experiment (Supplementary Figure S4).
Figure 3. NH₄⁺ facilitates ASO gene silencing in cancer cells. (A) Treatment with NH₄⁺ facilitated ASO silencing of targeted gene protein expression in LNCaP cells. Cells were treated with [1 μM] AR-ASO, [1 μM] BCL2-ASO, or [1 μM] β-Cat-ASO, with or without [5 mM] NH₄⁺, for 3 days. (B) Titration of AR-ASO [0.1–1 μM], in the presence of [5 mM] NH₄⁺ (lanes 3–6) in LNCaP cells. Lane 2 is AR-ASO [1 μM] without NH₄⁺. (C) NH₄⁺ facilitated ASO silencing of targeted mRNA. LNCaP cells were treated with [1 μM] AR-ASO with or without [5 mM] NH₄⁺ for 24 h; total RNA was then harvested for AR mRNA RT-PCR. The non-treated cells were defined as control cells. Cells treated with AR ASO but without NH₄⁺ were used as the comparator group for the treatment. Values were normalized to actin mRNA expression and expressed as the mean ± SD, n = 3. Adjusted **p-value < 0.003, Dunnett’s t-test. (D–E) NH₄⁺ enhanced the efficacy of a β-Cat-ASO in the colon cancer cell lines HCT 116 (D) and SW 480 (E). HCT 116 cells were treated with [1 μM] β-Cat-ASO with or without [5 mM] NH₄⁺ for 2 days; SW 480 cells were treated with 0.25 μM β-Cat-ASO with or without 2 mM NH₄⁺ for 2 days. α-Tubulin was used as control.

Figure 4. NH₄⁺ enhances ASO-directed gene silencing in transfection-resistant T cell lines. (A) Treatment with NH₄⁺ increased the efficacy of a BCL2-ASO in transfection-resistant Jurkat T cells. Cells were transfected with [50] or [100 nM] BCL2-ASO complexed with Lipofectamine® 3000 (lane 2 and 3), or treated with [1] or [2 μM] BCL2-ASO (as indicated) with or without [5 mM] NH₄⁺ (lane 4–7) for 2 days, prior to being harvested for Western blots. α-Tubulin was used as control. (B) Treatment with NH₄⁺ increased BCL2-ASO efficacy in CEM T cells. Cells were treated with the indicated concentrations of the BCL2-ASO with or without [5 mM] NH₄⁺ for 2 days, prior to Western blotting. α-Tubulin was used as control.
Figure 5. NH₄⁺ enhances gene suppression by two ONs delivered simultaneously. (A) NH₄⁺ simultaneously facilitated both BCL2-ASO-mediated BCL2 silencing and SSO-654-mediated EGFP expression in HeLa EGFP-654 cells [compare lane 8 to lane 5 (EGFP) and 6 (BCL2)]. Cells were treated with [0.5 μM] BCL2-ASO, [0.5 μM] SSO-654, or a combination of [0.5 μM] of both, in the presence or absence of [5 mM] NH₄⁺ for 2 days, prior to Western blotting. α-Tubulin was used as control. (B) The effect of SSO-654 titration ([1, 2 and 5 μM]) on BCL2-ASO-directed silencing in the presence of [5 mM] NH₄⁺. HeLa EGFP-654 cells were treated with the indicated SSO-654 and BCL2-ASO, with or without [5 mM] NH₄⁺, for 2 days prior to Western blotting. α-Tubulin was used as control. (C) The effects of increasing concentrations of BCL2-ASO [1, 2 and 5 μM] (BCL2-1, BCL2-2, and BCL2-5, as indicated) on SSO-654 gene silencing in the presence of [5 mM] NH₄⁺ in HeLa EGFP-654 cells. HeLa EGFP-654 cells were treated for 2 days prior to flow cytometry. The upper profiles and graph represent the combined flow cytometric analyses of three independent experiments. The y axis of the graph indicates the MFI of the cells. Data are expressed as the mean ± SD, n = 3. Increasing concentrations ([1, 2 and 5 μM]) of the BCL2 ASO do not dramatically affect the potency of SSO-654, except for the highest [5 μM] concentration [compare the third, fourth and fifth bar graphs to the second bar graph (no NH₄⁺) or the seventh, eighth and ninth bar graphs (with NH₄⁺)] to the sixth bar graph). The non-treated EGFP-negative cells were defined as control cells. Cells treated with SSO0-654 with and without NH₄⁺ were used as the comparator group for each additional treatment with or without NH₄⁺, respectively. Adjusted P-value of 0.08 [1 μM] BCL2, adjusted ***P-values < 0.001 [2 μM] and [5 μM] BCL2, adjusted P-value of 0.91 [1 μM] BCL2 + NH₄⁺, adjusted *P-values < 0.05 [2 μM] BCL2 + NH₄⁺, and adjusted ***P-values < 0.001 [5 μM] BCL2 + NH₄⁺ (Dunnett’s t-test). (D) NH₄⁺ simultaneously facilitates both β-Cat- and BCL2-ASO mediated β-catenin and BCL2 silencing, respectively, in HCT 116 cells. HCT 116 cells were treated with [1 μM] β-Cat-ASO and [1 μM] BCL2-ASO, or a combination of both, with or without [5 mM] NH₄⁺ as indicated, for 2 days.

Ammonium ion facilitates ON function, possibly by affecting the endosomal pathway.

To understand how NH₄⁺ facilitates ASO and SSO activity, we first examined whether it increased ON uptake in cells. As shown in Figure 6A, NH₄⁺ [5 mM] did not significantly affect the cellular uptake of a Cy5-labeled ASO (Cy5-ASO) in LNCaP cells (P = 0.98), suggesting that the activity of
NH₄⁺ occurs intracellularly. NH₄⁺ is known to inhibit endosomal maturation and lysosome fusion (21,22,28). Our data are not inconsistent with the idea previously demonstrated that the ammonium ion inhibits the fusion of endosomes with lysosomes (21). It is also possible that an NH₄⁺-induced endosomal swelling could contribute to the egress of ONs. However, other mechanisms may be involved, and without additional data, no conclusions can be drawn.

As noted above, endosomal maturation plays a critical role in ON delivery within cells after PS ON uptake via adsorptive and/or fluid phase endocytosis, as it has been found that 50% of the internalized ON is associated with lysosomes and other vesicular structures (29). Two other inhibitors of endosomal maturation/fusion, Ambroxol (Amb), and cyclohexylamine (CHA) (Supplementary Figure S5) in addition to OA (Supplementary Figure S5), which stimulates endosomal maturation, also significantly facilitated the ability of SSO-654 to promote EGFP expression in HeLa EGFP-654 cells when delivered by gynosis (Figure 6B, all with adjusted P-values < 0.01, and Supplementary Figure S6A and B, all with adjusted P-values < 0.001), indicating that enhancement of ON efficacy can occur at the level of the endosome. It is noteworthy that the latter compounds are organic amines; however, their pKas [8.25 (30) or 9.1 for Ambroxol (source DrugBank; ChemAxon), 10.62 for cyclohexylamine [31]; see Appendix G], are, like ammonia’s pKa (9.26) [31]; see Appendix G], are too high for any of these small molecules to reach the endosome in the unprotonated state. Therefore, they cannot act as intra-endosomal proton sponges.

Ammonium ion and As III synergistically facilitates the ability of SSO-654 to promote EGFP expression in HeLa EGFP-654 cells

In a recent study we found that As III facilitates SSO potency (Castanotto et al., submitted, and Figure 7A–D and Supplementary S8A; compare As III/SSO-654 with SSO-
Figure 7. NH$_4^+$ and As III are synergistic in facilitating SSO ability to promote EGFP expression. (A and B) Cells were treated with [1 μM] SSO-654, and [0.5 μM] As III, [5 mM] NH$_4^+$, or both as indicated for 2 days prior to microscopy and Western blotting. Scale bar = 100 μm. (C and D) Representative experiment showing the analysis of flow cytometric data obtained from HeLa EGFP-654 cells treated as in A and B. The profiles shown (C) are typical of three independent experiments. The y axis of the graph (D) represents EGFP MFIs. The non-treated EGFP-negative cells were defined as control cells. Cells treated with SSO-654 were used as the comparator group for each additional treatment. Data are represented as the mean ± SD, n = 3. Adjusted *P-value < 0.03 (SSO-654 + As III) and ***P-values < 0.001 for all other groups (Dunnett’s t-test). (E) Synergistic effect of a fixed molar ratio As III/NH$_4^+$ combination on SSO-654-mediated EGFP expression in HeLa EGFP-654 cells. The combination index (CI) plot was generated from the data in the Supplementary Table S1 and CompuSyn software (24). CI = 0.34 at the 50% effect (Fa 0.5 = 0.34); CI = 0.20 at the 75% effect (Fa 0.5 = 0.20).

654) in a dose-dependent manner (Supplementary Figure S8A). However, As III unlike NH$_4^+$, did not significantly increase β-catenin- or BCL2-ASO potency in HCT 116 or Jurkat T cells (Supplementary Figure S8B, compare lane 6 with lane 4). These findings support our observation that As III triggers the shuttling of the ON into the nuclear compartment (Castanotto et al., submitted). Since NH$_4^+$ seemed to enhance release of ON into the cytoplasm, the combination of the two small molecules could further augment the activity of the SSO in the nucleus. As shown in Figure 7A–D (fluorescence microscopy, Western blotting for EGFP, and flow cytometry respectively), the combination of NH$_4^+$, As III and SSO-654 significantly increased EGFP expression in HeLa EGFP-654 cells with limited toxicity (Supplementary Figure S7), compared with either the combination of NH$_4^+$ and SSO-654, or of As III and SSO-654. The combination of NH$_4^+$ and OA or Amb, are shown in Supplementary Figure S9.

By western blotting, cells treated with NH$_4^+$ + As III manifested more than a ten-fold increase in SSO-654-mediated EGFP expression than treatment with the SSO-654 alone. This is compared to a 3- to 5-fold upregulation of SSO-654-mediated EGFP expression by either NH$_4^+$ or As III as single agents. This was evaluated by western blotting (Figure 7B) and by flow cytometry (Figure 7C and D). To investigate whether these two small molecules in combination produced a synergistic effect on SSO activity, we performed a dose-response study (see Supplementary Table S1). Quantitative analysis was performed by flow cytometry, which allowed us to produce a combination index (CI) plot using the Chou–Talalay method (24), in which the line parallel to the x-axis (CI = 1, Figure 7E) represents the divide between synergism (CI < 1) and antagonism (CI > 1) for the two drugs being analyzed in combination. A CI < 1 demonstrates drug synergism, which increases as CI decreases. The two most relevant points in our experiment, performed at fractional activity (Fa), = 0.5 and 1.0, fall well below CI = 1 (Figure 7E; note that the lower ammonium + As III concentrations produced essentially only basal SSO-654 activity). Therefore, the combination of NH$_4^+$ and As III was synergistic with respect to the facilitation of SSO-654 function in HeLa EGFP-654 cells.
Figure 8. Schematic representation of some potential mechanisms leading to synergistic NH$_4^+$ and As III facilitation of ON-directed gene silencing. Gymnotically delivered ONs are taken up into cells via the process of endocytosis, and are initially localized in early endosomes (EE). During the process of vesicular acidification (21,22,28), these EEs matures into the late endosomes/multivesicular bodies (LEs/MVBs), which will fuse with nuclease-rich lysosomes where ONs are sequestered/degraded. NH$_4^+$ inhibits endosomal acidification, causes endosomal swelling, and inhibits fusion of LEs with lysosomes. Prolonging maturation of the endosomes in addition to inhibiting fusion between later endosomes and lysosomes may facilitate ON escape from endosomes into the cytosol. As III, which does not appear to affect the release of ONs from the endosomal compartment, facilitates the entry of cytoplasmic ON into the nuclear compartment via inducing a cellular stress response (Castanotto et al., submitted). The combination of NH$_4^+$ and As III is synergistic with respect to the nuclear function of SSO-654, perhaps via these proposed mechanisms.

DISCUSSION

In this work, we elected to study the effects of NH$_4^+$ on the activity of ONs because of our previous results (20), which highlighted the importance of endosomal maturation for ON activity and because it has previously been shown that NH$_4^+$ affects the maturation and outcomes of late endosomes (21–23). After cell surface adsorption, or through fluid phase endocytosis, ONs become localized inside the cell in early endosomes. The maturation of early to late endosomes (LEs/MVBs) is at least in part under the control of PKC-α/H$_9251$. Blocking PKC-α expression by a variety of methods leads to a marked diminution of ASO gene silencing, suggesting that ONs, at least in part, exit the endosomal pathway at the level of the LE/MVBs. In this context, it is of interest that Ago-2, one of the proteins that interacts with ONs (32) and shuttles the ONs to the cell nucleus (Castanotto et al., submitted), can physically interact with the LE (33). (However, it still remains unclear how negatively charged PS ONs, when delivered by gymnosis, can penetrate the hydrophobic late endosomal membrane.)

LEs/MVBs can also fuse with lysosomes; we and others (32,34) have demonstrated SSO accumulation in lysosomes, a process deemed to be non-productive with respect to ON activity. The ability of NH$_4^+$ to block/slow the fusion of LEs/MLVs with lysosomes (21–23) might allow the endosomal cargo to be retained for longer times in the LE/MVB, increasing its ability to exit the endosomal pathway. The exit of ONs from the endosome might also be aided by the ability of NH$_4^+$ and of other lysosomotropic weak bases, to also cause endosomal swelling (35,36). The way that endosomal swelling is produced by NH$_4^+$ is complex, but cannot be due to proton sponge effects, as the pK$_a$ of NH$_4^+$ = 9.26 (31), two orders of magnitude higher than the intracellular pH. The endosomal swelling effects of NH$_4^+$ may mimic those of the so-called cell penetrating peptide-ONs, in which the peptide moieties are usually short (9-30 amino acids) polypeptides that are often replete with arginines and lysines (37), both of which are organic amines.

However, the mechanism of action of NH$_4^+$, regardless of how it enhances ONs (ASO and SSO) function, appears to be different than that of As III (a mechanistic model is suggested in Figure 8). The latter enhances ON function in the nucleus but not in the cytoplasm (Supplementary Figure S8), while NH$_4^+$ enhances function in both cellular compartments, possibly by increasing the total amount of ON released into the cytoplasm. This is in accord with our previous observations that As III, through induction of cellular stress, facilitates shuttling of ONs from the cytoplasm to the nucleus (Castanotto et al., submitted). In contrast to As III, under the conditions of our experiments, NH$_4^+$ [5 mM], does not appear to induce a cellular stress response and thus the formation of a ONs-binding stress-induced response complex (SIRC), which consists of Ago-2, nucleolin, and Yb-1, in addition to other proteins (Castanotto et al., submitted). Furthermore, the data demonstrating (Figure 7) that the higher concentrations of NH$_4^+$/As III are synergistic, as defined by the combination index (CI)
method of Chou and Talalay (24), also suggest that these small molecules augment ON activity by different mechanisms. Of note, we performed a gene expression profiling study with Jurkat T cells under our experimental conditions. We found that NH4+ treatment did not detectably affect the expression of known genes involved in the endocytosis or in the endosome/lysosome pathway (not shown). This finding is consistent with our model that NH4+ regulates the biogenesis and maturation of endosome/lysosome at the biochemical level. The only detectable changes in gene expression clustered with those involved in lipid biosynthesis and metabolic pathways (not shown), which is consistent with previously published gene expression profiles following NH4+ treatment (38). The possibility that the positively charged NH4+ augments binding of an ON to its mRNA target by charge masking may be discounted, as experiments examining the Tm of DNA/RNA duplexes in the presence or absence of 5 mM NH4+ demonstrated no difference (not shown).

In summary, the combination of As III, which has previously been shown to be a potential anti-leukemia drug (39–42), and/or NH4+ (or other organic amines such as Amb., or other molecules such as OA that act on the endosomal maturation pathway), is an easy, inexpensive, non-toxic and effective way to improve SSO and ASO activity after their delivery by ngnosis, even in non-attached, difficult to transfect cells. Ca2+ has been shown to improve ON function by increasing its cellular uptake (43). However, the toxicity of Ca2+, which at the required 9 mM concentration is not clinically viable, makes the As III/NH4+ combination more appealing. Like As III, NH4+, which possesses expectorant and diuretic properties, is an FDA-approved drug that has been used for the treatment of human diseases. Most likely, the synergistic increase in function is produced because these agents, as demonstrated by the Combination Index plot, appear to be active by different mechanisms: NH4+ could possibly be acting at the level of the endosomes (as suggested in Figure 8), while As III seems to induce a cellular stress reaction that promotes cytoplasmic-nuclear ON shuttling (Castanotto et al., submitted). As these compounds have already found a niche in the clinic, these mechanisms can be further explored with the ultimate goal of enhancing ON activity for therapeutic use.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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