

# Cell-penetrating peptides as transporters for morpholino oligomers: effects of amino acid composition on intracellular delivery and cytotoxicity

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## ABSTRACT

Arginine-rich cell-penetrating peptides (CPPs) are promising transporters for intracellular delivery of antisense morpholino oligomers (PMO). Here, we determined the effect of L-arginine, D-arginine and non- $\alpha$  amino acids on cellular uptake, splice-correction activity, cellular toxicity and serum binding for 24 CPP-PMOs. Insertion of 6-amino hexanoic acid (X) or  $\beta$ -alanine (B) residues into oligoarginine R<sub>8</sub> decreased the cellular uptake but increased the splice-correction activity of the resulting compound, with a greater increase for the sequences containing more X residues. Cellular toxicity was not observed for any of the conjugates up to 10  $\mu$ M. Up to 60  $\mu$ M, only the conjugates with  $\geq 5$  Xs exhibited time- and concentration-dependent toxicity. Substitution of L-arginine with D-arginine did not increase uptake or splice-correction activity. High concentration of serum significantly decreased the uptake and splice-correction activity of oligoarginine conjugates, but had much less effect on the conjugates containing X or B. In summary, incorporation of X/B into oligoarginine enhanced the antisense activity and serum-binding profile of CPP-PMO. Toxicity of X/B-containing conjugates was affected by the number of Xs, treatment time and concentration. More active, stable and less toxic CPPs can be designed by optimizing the position and number of R, D-R, X and B residues.

## INTRODUCTION

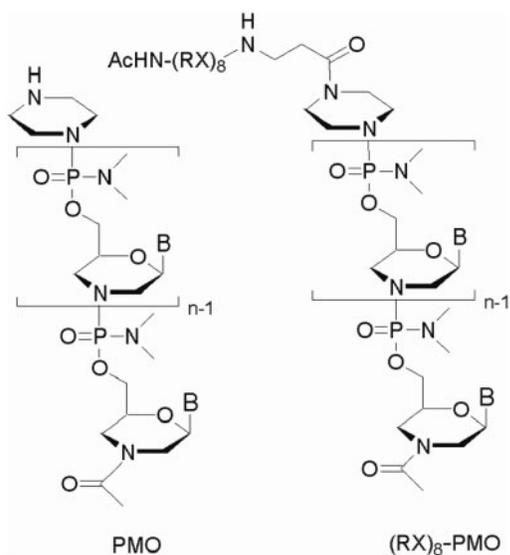
Steric-blocking antisense oligonucleotides (AOs) are considered potential therapeutics for genetic diseases such as Duchenne muscular dystrophy (DMD) and  $\beta$ -thalassemia. For their potential to be realized, however, the AOs must be effectively delivered to cell nuclei.

Cationic lipoplex- or PEI-based transfection methods used to deliver charged AOs are not suitable for the delivery of uncharged AOs such as phosphorodiamidate morpholino oligomers (PMO, Figure 1) (1) and peptide nucleic acids (PNAs) (2). Conjugation of PMO to short CPPs is a good method to enhance the cytoplasmic and nuclear delivery of PMO because the conjugates are simple to use and because the short peptides and their AO conjugates can be easily manufactured and characterized in a quality-controlled manner. Examples of well-studied CPP-PMO conjugates include those with Tat and oligoarginine peptides (3,4).

Important considerations in the design of effective CPPs include the ability to deliver AO efficiently, stability in living systems and toxicity. We have reported that Tat and oligoarginine peptides are not stable in human serum (5), and are therefore ill-suited for *in vivo* applications. Oligoarginine peptides incorporating non- $\alpha$  amino acids have been proven superior to oligoarginine alone. CPPs containing 6-amino hexanoic acid (X) and  $\beta$ -alanine (B) were more stable in human serum than Tat or oligoarginine peptides (5). A CPP-PMO conjugate, (RXR)<sub>4</sub>-PMO, has been shown to be more efficient in the correction of pre-mRNA mis-splicing (6) and in inhibition of the replication of mouse hepatitis virus *in vivo* (7) than an oligoarginine peptide. In addition, (RXR)<sub>4</sub>-PMO conjugates have been shown to cause effective exon skipping in muscle cells from DMD dogs (8), in human muscle explants (9) and in *mdx* mice (10), as well as inhibiting the replication of various viruses in cell cultures (7,11–13) and in mice (7,13).

The above studies have helped make it clear that unnatural amino acids can confer enhanced stability and activity, and therefore improve the potential of CPPs to deliver therapeutic PMO. In pursuit of CPPs with improved characteristics, we have carried out a structure-activity relationship study to investigate the effects of unnatural amino acid insertions in oligoarginine peptides on cellular delivery, nuclear antisense activity, toxicity and serum-binding characteristics of the resulting CPP-PMO

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**Figure 1.** Structures of PMO and  $(RX)_8$ -PMO conjugate.

conjugates. The unnatural amino acids studied here are X, B and D-arginine (r). We chose to study the X amino acid based on the successes of the  $(RXR)_4$  CPP in several studies as shown in the previous paragraph. B and r amino acids were chosen because they have good enzymatic stability (5). The CPPs are (i) the oligoarginine sequences,  $R_8$  and  $R_9$ , (ii) sequences with RXR, RX and RB repeats, as well as various combinations thereof, and (iii) sequences containing D-arginine,  $r_8$ ,  $(rX)_8$ ,  $(rXR)_4$ ,  $(rXr)_4$  and  $(rB)_8$ . The **CPP-PMO** conjugates were evaluated for their relative (a) cellular uptake, as determined by flow cytometry, (b) antisense activity, as determined by a splice correction assay (13) and (c) cellular toxicity, as determined by MTT cell viability, propidium iodide membrane integrity and hemolysis assays, as well as by microscopic imaging.

## MATERIALS AND METHODS

### Synthesis of **CPP-PMO** conjugates

CPP nomenclature and sequences are listed in Table 1. Chemical structures of PMO and  $(RX)_8$ -PMO are shown in Figure 1. The antisense PMO (CCT CTT ACC TCA GTT ACA) is designed to target a  $\beta$ -thalassemic mutant splice site present in the human  $\beta$ -globin intron 2 of a positive-readout antisense activity assay system (13) as described in the Results section. Synthesis of PMO, described previously (15,16), and the CPPs, using standard Fmoc chemistry (17), were performed at AVI BioPharma, achieving purities of >90% as determined by HPLC and mass spectrometry analysis. Conjugation of a CPP to a PMO through an amide linker, described previously (6), was followed with an additional purification step to remove nonconjugated peptide. Samples were loaded on source 30S resin (Amersham Biosciences, Pittsburgh, PA) in a 2 ml Biorad (Hercules, CA) MT2 column at 2 ml/min with running buffer A (20 mM  $Na_2HPO_4$ , 25% acetonitrile, pH 7.0) and purified into 45-s fractions with 0–35%

**Table 1.** Names and sequences of the CPPs

Name	Sequence*	# of X
Oligoarginine		
$R_8$	RRRRRRRRXB	1
$r_8$	rrrrrrrrXB	1
$R_9$	RRRRRRRRXB	1
RX, RXR and RB panels		
$(RX)_8$	RXXRXXRXXRXXRXXB	8
$(rX)_8$	rXrXrXrXrXrXrXB	8
$(RX)_7$	RXXRXXRXXRXXRXXB	7
$(RX)_5$	RXXRXXRXXB	5
$(RX)_3$	RXXRXXB	3
$(RXR)_4$	RXXRXXRXXRXXB	5
$(rXR)_4$	rXRrXRrXRrXrXB	5
$(rXr)_4$	rXrXrXrXrXrXB	5
$(RB)_8$	RBRBRBRBRBRBRBB	0
$(rB)_8$	rBrBrBrBrBrBrBB	0
$(RB)_7$	RBRBRBRBRBRBB	0
$(RB)_5$	RBRBRBRBB	0
$(RB)_3$	RBRBRBB	0
RX, RB, RXR and RBR mix panel		
2	RBRBRBRBRBRBRXB	2
3a	XRBRBRBRBRBRBRXB	3
3b	RXXRBRBRBRBRXB	3
3c	RBRBRBRBRBRBRXX	3
3d	RXXRBRBRBRBRBRXB	3
4a	RBRBRBRBRBRBRXB	4
4b	RBRXRBRBRBRBRXB	4
4c	RXXRXXRBRBRBRXB	4

\*The sequences of peptides are written from N to C terminus. R = arginine, r = D-arginine, X = 6-amino-hexanoic acid, B =  $\beta$ -alanine. Each peptide had an acetyl group at the N-terminus and a carboxyl group at the C-terminus.

buffer gradient (buffer B: 1.5M NaCl, 20 mM  $Na_2HPO_4$ , 25% acetonitrile, pH 7.0) over 60 min, using a Biorad BioLogic low pressure chromatography system. The desired fraction was desalted by a method described previously (6). HPLC and MS analyses revealed that the final product contained >90% CPP conjugated to full-length PMO, with the balance composed of CPP conjugated to incomplete PMO sequence, nonconjugated full-length or incomplete PMO.

### Cell culture

The HeLa pLuc705 (pLuc705) (14) cell line was obtained from Gene Tools, LLC (Philomath, OR). Human liver cell line HepG2 was from American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in RPMI 1640 medium supplemented with 2 mM L-Glutamine, 100 U/ml penicillin and 10% fetal bovine serum (FBS) (HyClone, Ogden, UT) at 37°C in a humidified atmosphere containing 5%  $CO_2$ . All treatments were carried out in OptiMEM medium (Gibco, Inc., Carlsbad, CA.) with or without FBS.

### Cell uptake assay

pLuc705 cells were seeded 20 h prior to treatment in 12-well plates at 100 000 cells/well. Cells were treated with 2  $\mu$ M fluorescein-tagged **CPP-PMO** conjugates for 24 h. After treatment, cells were washed with 500  $\mu$ l of

cold PBS, incubated with 400  $\mu$ l of trypsin at 37°C for 10 min and combined with 500  $\mu$ l medium containing 10% FBS. Cells were spun down at 1000g for 3 min, washed with 500  $\mu$ l cold PBS twice, resuspended in 200  $\mu$ l of PBS containing 1% FBS and analyzed by a FC-500 Beckman Coulter cytometer (Fullerton, CA). Data was processed using FCS Express 2 (De Novo Software, Thornhill, Ontario, Canada).

#### Nuclear activity assay

pLuc705 cells were seeded 20 h before treatment in 48-well plates at 30 000 cells/well. Cells were washed with 200  $\mu$ l medium and treated with **CPP-PMO** conjugates for 24 h. Cells were washed twice with 200  $\mu$ l PBS (HyClone, Ogden, UT), and incubated with 100  $\mu$ l of cell lysis buffer (Promega, Madison, WI) at 4°C for 30 min. Cell lysate was separated from the cell debris by centrifugation at 1000g for 10 min at 4°C. Luciferase levels were determined by mixing 30  $\mu$ l of cell lysate and 50  $\mu$ l of luciferase assay reagent (Promega) and measuring subsequent light production using a Flx 800 microplate fluorescence/luminescence reader (Bio-tek, Winooski, VT). The relative light units (RLU) per sample were normalized to microgram of sample protein as determined by the bicinchoninic acid method, following the manufacturer's procedure (Pierce, Rockford, IL).

#### Cell viability assay and microscopy

pLuc705 cells were seeded 20 h before the treatment in 96-well plates at 9000 cell/well and then treated with the conjugates. The microscopic phase images of treated cells were visualized by a Nikon Diaphot inverted microscope (Melville, NY), captured by an Olympus digital camera and processed by the Magnafire software (Optronics, Goleta, CA). After imaging the cells, the cell viability was determined by the methylthiazolotetrazolium assay (MTT, Sigma, St. Louis, MO) assay. MTT solution (5 mg/ml) was added to the treatment medium to a final concentration of 0.5 mg/ml and incubated for 4 h at 37°C. 85% of the media of each well was then replaced with DMSO containing 0.01M HCl and further incubated for 10 min at 37°C and the absorbance measured at 540 nm. Percent cell viability was determined by normalizing the absorbance of each treated sample to the mean of untreated samples.

#### Propidium iodide membrane integrity assay

pLuc705 cells were seeded 20 h before treatment in 12-well plates at 100 000 cells/well. Cells were treated by removing the medium, washing with 500  $\mu$ l PBS and incubating with medium containing **CPP-PMO** conjugates. Treatment medium was collected in tubes and cells were washed with PBS once, and then treated with 400  $\mu$ l of 10% trypsin for 10 min at 37°C. Trypsin was neutralized with 500  $\mu$ l of the serum containing medium. Cells were transferred to the tubes containing previously collected treatment medium, pelleted by centrifugation at 1000g for 5 min, washed with PBS once, and re-suspended in 200  $\mu$ l of 0.05  $\mu$ g/ml propidium iodide (PI) in PBS. Cells were further incubated

at 37°C for 15 min and analyzed by the Beckman Coulter cytometer (30 000 events/sample collected).

#### Hemolysis assay

The hemolytic activities of the conjugates were determined in fresh rat blood according to a method described elsewhere (18).

## RESULTS

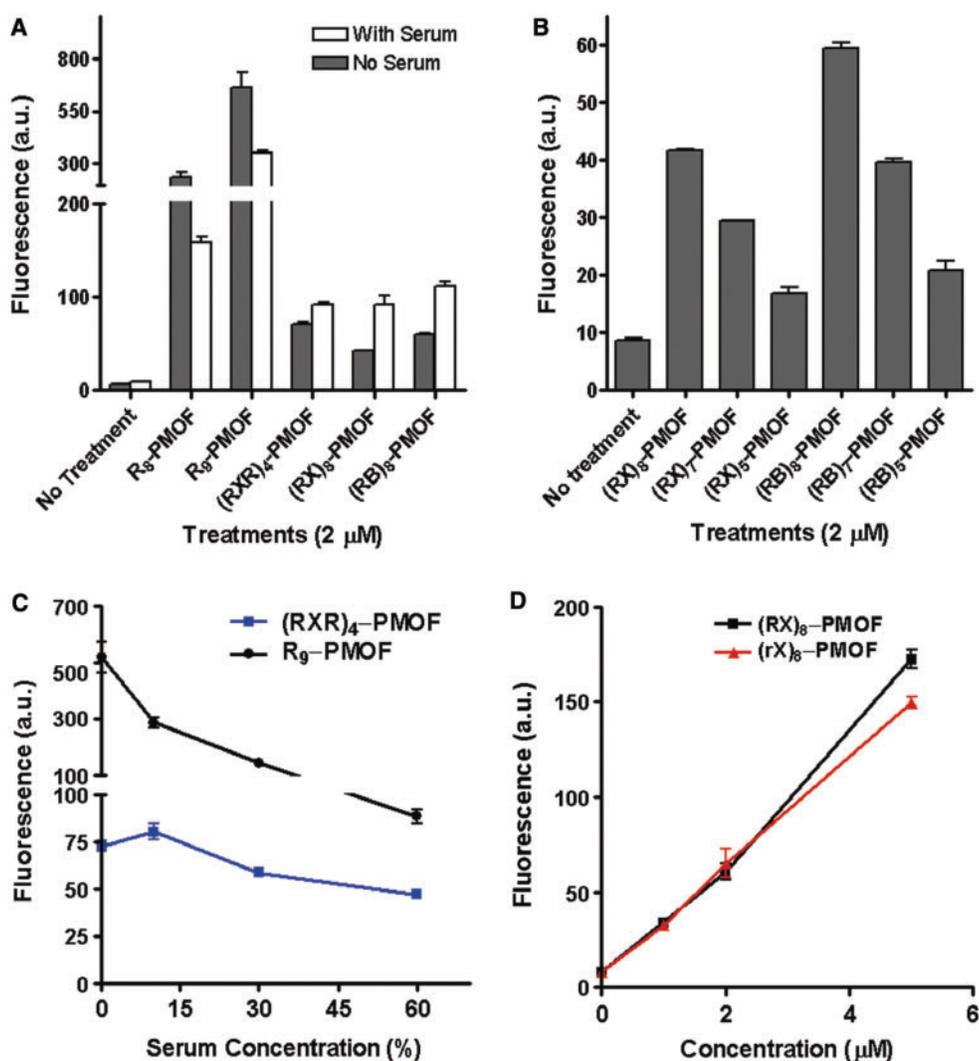
### Cellular Uptake

Cellular uptake of **CPP-PMO** conjugates was investigated using the 3'-carboxyfluorescein-tagged PMO (PMOF) and flow cytometry. We chose 2  $\mu$ M as the treatment concentration because none of the conjugates caused any detectable cytotoxicity at this concentration, as demonstrated by the MTT and PI uptake assays. After treating with the conjugates, cells were treated with trypsin (19) to remove membrane-bound conjugates. We found that a heparin sulfate washing step prior to trypsin treatment did not remove additional membrane-bound conjugates but caused some cellular toxicity (data not shown); therefore, only the trypsin treatment step was used in this study.

To determine the effect of serum on cellular uptake of the various conjugates, uptake evaluation assays were carried out in the medium containing various concentrations of, or in the absence of, serum. Cellular uptake of **CPP-PMOF** conjugates increased with the number of arginines and decreased with the X and/or B residue insertion (Figure 2A and B). The oligoarginine  $R_9$ -PMOF had a mean fluorescence (MF) of 662, nearly 3-fold higher than the 234 produced by  $R_8$ -PMOF, indicating that a difference of a single arginine can make a substantial difference in the biological properties of a CPP. Insertion of an X or B residue in the  $R_8$  sequence reduced the MF from 234 of  $R_8$ -PMO to 42, 70 and 60 of  $(RX)_8$ -,  $(RXR)_4$ - and  $(RB)_8$ -PMOF, respectively (Figure 2A). The number of RX or RB repeats affected cellular uptake, with conjugates having fewer RX or RB repeats generating lower MF (Figure 2B).

While the addition of 10% serum to the medium caused a decrease in the uptake of the  $R_8$ - or  $R_9$ -PMOF conjugates, it increased the uptake of conjugates containing RX, RB or RXR motifs (Figure 2A and C). Serum reduced the MF of  $R_9$ - and  $R_8$ -PMOF from 662 and 234 to 354 and 158, respectively, and increased the MF of  $(RX)_8$ -,  $(RXR)_4$ - and  $(RB)_8$ -PMOF from 41, 70 and 60 to 92, 92 and 111, respectively. These differences were statistically significant (Figure 2A). However, higher serum concentrations (30 and 60%) decreased the uptake of  $(RXR)_4$ -PMOF and oligoarginine-PMOF (Figure 2C).

Arginine stereochemistry (D versus L) had little effect on the uptake of **CPP-PMOF** conjugates. We compared the MF of  $R_8$ -,  $(RB)_8$ - and  $(RX)_8$ -PMOF with their respective D-isomer conjugates,  $r_8$ -,  $(rB)_8$ - and  $(rX)_8$ -PMOF and found that there was no significant difference between each pair, as shown in Figure 2D for the  $(RX)_8$ - and  $(rX)_8$ -PMOF pair.



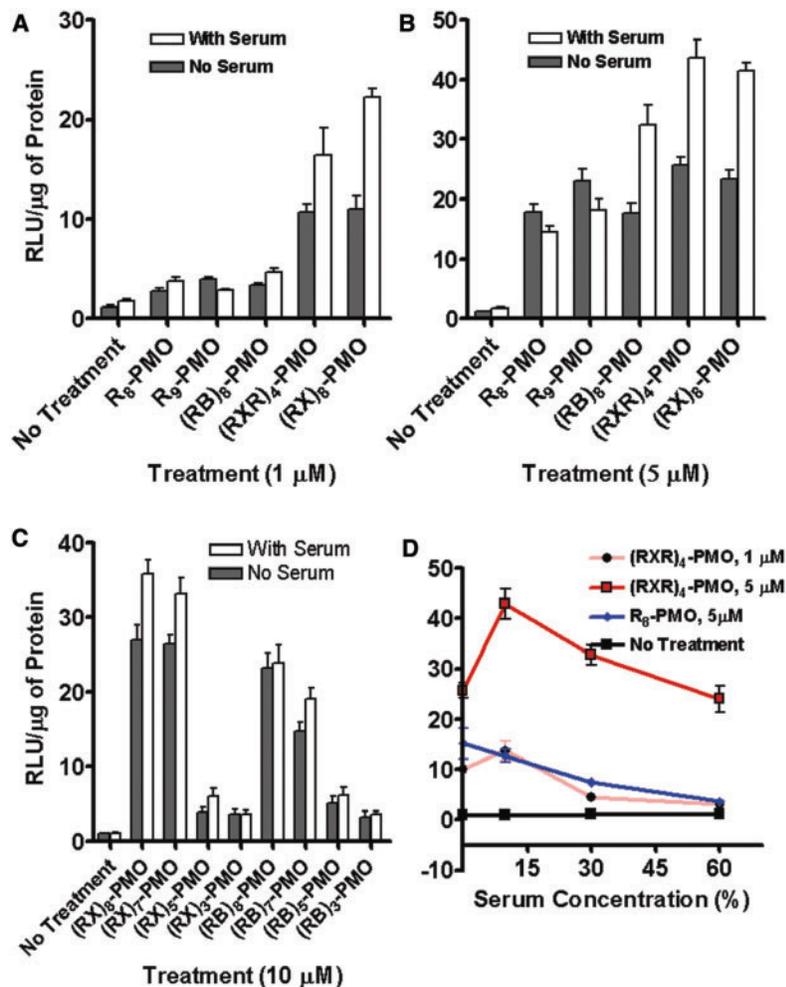
**Figure 2.** Cellular uptake of the CPP-PMOF conjugates. pLuc705 cells were treated with carboxyfluorescein-tagged PMOs conjugated to CPPs in OptiMEM with or without 10% serum for 24 h, followed by flow cytometry analysis. Data are presented as mean fluorescence (MF)  $\pm$  SD of six data points from two independent experiments. (A) Cells treated with 2  $\mu$ M of R<sub>8</sub>-, R<sub>9</sub>-, (RXR)<sub>4</sub>-, (RX)<sub>8</sub>- or (RB)<sub>8</sub>-PMOF conjugates. (B) Cells treated with 2  $\mu$ M of (RX)<sub>8</sub>-, (RX)<sub>7</sub>-, (RX)<sub>5</sub>-, (RB)<sub>8</sub>-, (RB)<sub>7</sub>- or (RB)<sub>5</sub>-PMOF conjugates in the absence of serum. (C) Cells treated with 2  $\mu$ M of (RXR)<sub>4</sub>- or R<sub>9</sub>-PMOF in media containing 0, 10, 30 or 60% serum. (D) Cells treated with (RX)<sub>8</sub>- or (rX)<sub>8</sub>-PMOF in media containing 10% serum.

### Nuclear antisense activity.

The effectiveness of each CPP-PMO conjugate was determined in a previously described splicing correction assay (14), considered a reliable method to assess nuclear antisense activity of a steric-blocking AO. This assay utilizes the ability of steric-blocking AOs to block a splice site created by a mutation in order to restore normal splicing. The luciferase coding sequence was interrupted by the human  $\beta$ -globin thalassemic intron 2 which carried a mutated splice site at nucleotide 705. HeLa cells were stably transfected with the plasmid therefore named as pLuc705 cell. In the pLuc705 system, steric-blocking AOs must be present in the cell nucleus for splicing correction to occur. Advantages of this system include the positive readout and high signal-to-noise ratio. [With this system the relative efficiencies of various CPPs to deliver an AO](#)

[with sequence appropriate for splice-correction to cell nuclei can be easily compared.](#)

*Oligoarginine, RX, RXR and RB panels.* The CPP conjugates with the highest nuclear antisense activities were (RXR)<sub>4</sub>- and (RX)<sub>8</sub>-PMO. Figure 3A and B show luciferase activity normalized to protein of cells treated with various conjugates at 1 and 5  $\mu$ M for 24 h. At both concentrations, (RX)<sub>8</sub>- and (RXR)<sub>4</sub>-PMO were more effective than the other conjugates tested, with the difference more prominent in serum-containing medium at 1  $\mu$ M than at 5  $\mu$ M. Cells treated with 1  $\mu$ M of either conjugate had luciferase activity 10–15-fold over the background while the remaining conjugates yielded about a 2–4-fold over the background (Figure 3A). At 5  $\mu$ M, all conjugates generated higher luciferase activity



**Figure 3.** Nuclear activity of **CPP-PMO** conjugates: *oligoarginine*, *RXR*, *RX* and *RB* panels. pluc705 cells were treated with the conjugates in OptiMEM medium with or without 10% serum for 24 h. Nuclear activity of a conjugate is indicated by relative luciferase activity (RLU) per microgram of protein. Data represent a mean  $\pm$  SD of 6–9 data points from three independent experiments. (A) Cells treated with 1  $\mu$ M of  $R_8$ –,  $R_9$ –,  $(RB)_8$ –,  $(RXR)_4$ – or  $(RX)_8$ –PMO. (B) Cells treated with 5  $\mu$ M of the same panel of conjugates. (C) Cells treated with 10  $\mu$ M of conjugates with 8, 7, 5 or 3 RX or RB repeating units. (D) Cells treated with  $(RXR)_4$ – or  $R_8$ –PMO at 1 or 5  $\mu$ M in 0, 10, 30 and 60% serum-containing medium.

than at 1  $\mu$ M, with  $(RX)_8$ –PMO and  $(RXR)_4$ –PMO again the most effective, followed by  $(RB)_8$ –PMO (Figure 3B). Figure 3C shows that at 10  $\mu$ M, the activity of RX or RB conjugates decreased as the number of RX or RB repeats in the CPP decreased. The peptides with 5 or 3 RX or RB repeats,  $(RX)_5$ ,  $(RX)_3$ ,  $(RB)_5$  or  $(RB)_3$ , generated much lower luciferase activity than those with 8 and 7 repeats.

**Number and position of X residues.** Having shown that  $(RB)_8$ –PMO had less activity than  $(RXR)_4$ –PMO or  $(RX)_8$ –PMO, we further investigated the effect of the number and position of X residues on the activity of conjugates. Eleven **CPP-PMO** conjugates containing 0, 2, 3, 4, 5 or 8 Xs were compared (Figure 4). Generally, CPPs containing a higher number of Xs had higher activities. At 2  $\mu$ M,  $(RX)_8$ –PMO (8 X residues) had the highest activity followed by  $(RXR)_4$ –PMO (5 X residues) and the conjugates with fewer Xs had lower activities. At 5  $\mu$ M, three conjugates containing 3 (3d), 4 (4c) and 8 ( $(RX)_8$ ) X

residues had the highest activities, suggesting that the position of X residues affects activity.

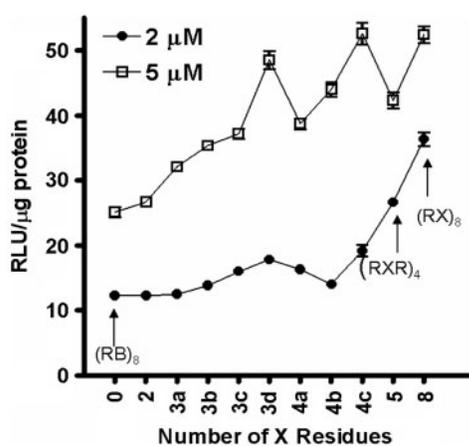
**L-Arginine versus D-arginine.** Arginine stereochemistry had little effect on the nuclear activity of the  $R_8$ – and  $(RB)_8$ –PMO conjugates but affected the  $(RX)_8$ –PMO (Figure 5). Replacement of the eight L-arginine residues in  $R_8$ – or  $(RB)_8$ –PMO with D-arginine residues did not change the luciferase activity generated over the 1–5  $\mu$ M (Figure 5A and B). However, the replacement did cause a small but statistically significant decrease in the activity for  $(RX)_8$ –PMO at 1  $\mu$ M ( $P = 0.03$ ) and 2  $\mu$ M ( $P = 0.01$ ) (Figure 5C).

**Serum effect on activity.** The effect of serum on the antisense activity of the conjugates depended on the CPP sequences, as shown in Figure 3A–D. Addition of 10% serum to the medium decreased the activity of oligoarginine–PMO conjugates ( $R_8$ –PMO and  $R_9$ –PMO) but increased activity of conjugates containing

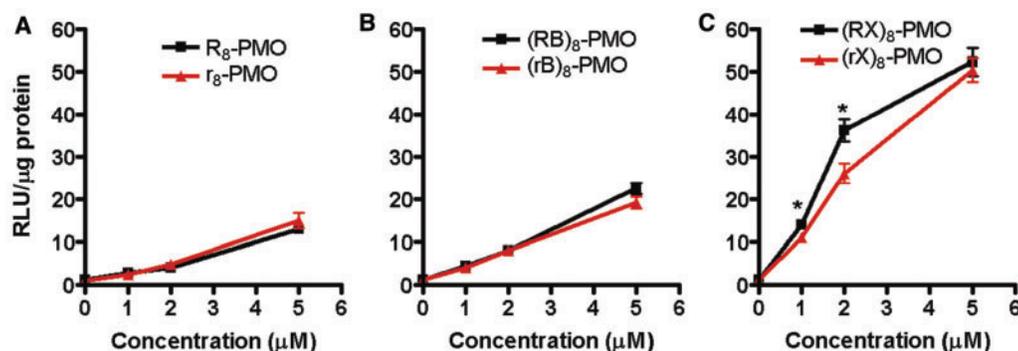
RXR, RX and RB repeats. The addition of 10% serum nearly doubled the luciferase activity of  $(RXR)_4$ -,  $(RX)_8$ - and  $(RB)_8$ -PMO at 5  $\mu$ M (Figure 3B). We further studied this effect for  $(RXR)_4$ -PMO up to 60% serum. While the activity almost doubled as the serum concentration increased from 0 to 10%, it gradually decreased as the serum concentration increased to 60%, at which activity was similar to that in 0% serum which was still significantly above the background. This 'up and down' profile was also observed with the 1  $\mu$ M  $(RXR)_4$ -PMO treatment. Unlike  $(RXR)_4$ -PMO, the luciferase activity of  $R_8$ -PMO or  $R_9$ -PMO (data not shown) only decreased as the serum concentration increased, with an approximately 30% reduction in 10% serum and no activity in 60% serum.  $R_8$ -PMO or  $R_9$ -PMO did not display any detectable activity at 1  $\mu$ M, regardless of the serum concentration (data not shown).

### Toxicity

The cellular toxicity of the various **CPP-PMO** conjugates was determined by MTT-survival, propidium iodine (PI)



**Figure 4.** Nuclear activity of **CPP-PMO** conjugates: number and position of X residues. pluc705 cells were treated with the conjugates having 0, 2, 3 (3a, 3b, 3c and 3d), 4 (4a, 4b and 4c), 5 and 8 X residues (see sequences in Table 1) in OptiMEM medium with 10% serum for 24h. Nuclear activity of a conjugate is indicated by relative luciferase activity (RLU) per microgram of protein. Data represent a mean  $\pm$  SD of 9–12 data points from four independent experiments.

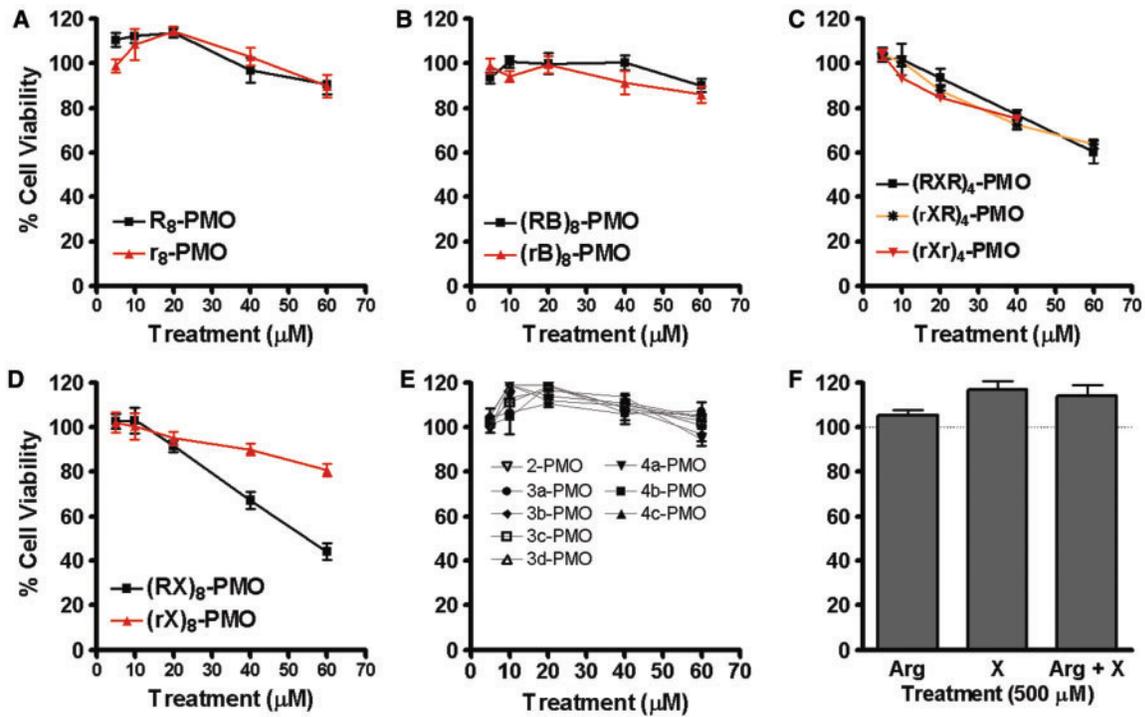


**Figure 5.** Nuclear activity of **CPP-PMO** conjugates: D-arginine versus L-arginine. pluc705 cells were treated with the indicated compounds at 1, 2 and 5  $\mu$ M in OptiMEM containing 10% FBS for 24h. Data represent a mean of 9–12 samples from four independent experiments. \* Indicates that difference in activity between the  $(RX)_8$  and  $(rX)_8$ -PMO conjugates were statistically significant.

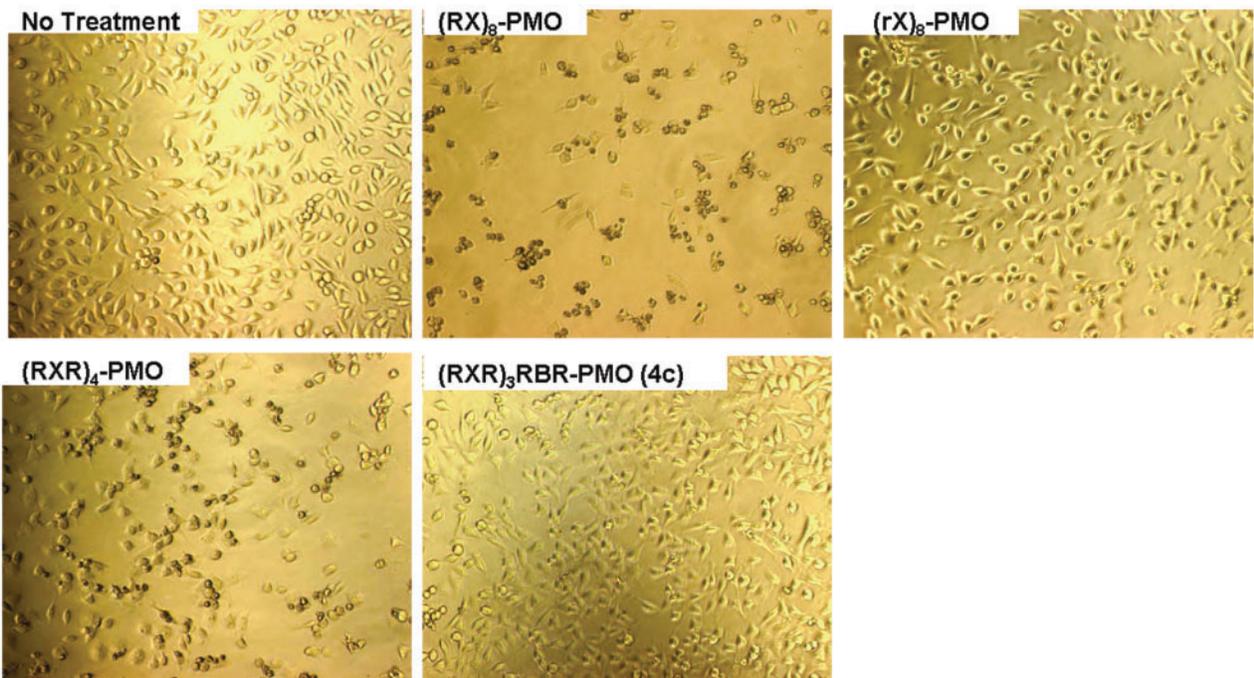
exclusion and hemolysis assays and microscopic imaging. The MTT and PI exclusion assays measure metabolic activity and membrane integrity of cells, respectively. The hemolysis assay determines compatibility with blood. Microscopic images were used to verify the MTT results and observe the general health of the cells.

**MTT assay.** pLuc705 cells were treated at concentrations ranging from 2 to 60  $\mu$ M for 24 h. As shown in Figure 6, all conjugates, except  $(RX)_8$  and  $(RXR)_4$ , had no toxicity at up to 60  $\mu$ M. Up to 10  $\mu$ M,  $(RX)_8$  and  $(RXR)_4$  conjugates exhibited no toxicity, at higher concentrations they reduced cell viability in a concentration-dependent manner, with  $(RX)_8$  being more toxic than  $(RXR)_4$  (Figure 6C and D). Replacement of L-arginine with D-arginine in  $R_8$ -,  $(RB)_8$ - and  $(RXR)_4$ -PMO did not change the viability profiles of these conjugates (Figure 6A–C). Surprisingly, the L $\rightarrow$ D replacement in  $(RX)_8$ -PMO decreased the toxicity. Cell viability with 60  $\mu$ M treatment was 40% for  $(RX)_8$ -PMO, but 80% for  $(rX)_8$ -PMO (Figure 6D). The eight conjugates containing fewer than 5 X residues did not inhibit cell proliferation up to 60  $\mu$ M (Figure 6E). Monomers of arginine or X, individually or in combination, at 500  $\mu$ M each, produced no inhibition of cell proliferation (Figure 6F). The toxicities of the **CPP-PMO** conjugates,  $(RXR)_4$ -PMO, 3d-PMO and 4c-PMO, were also evaluated in human liver HepG2 cells. We found that only  $(RXR)_4$ -PMO caused dose-dependent inhibition of cell proliferation while other two conjugates had no toxicity up to 60  $\mu$ M, the highest concentration tested in this study (data not shown).

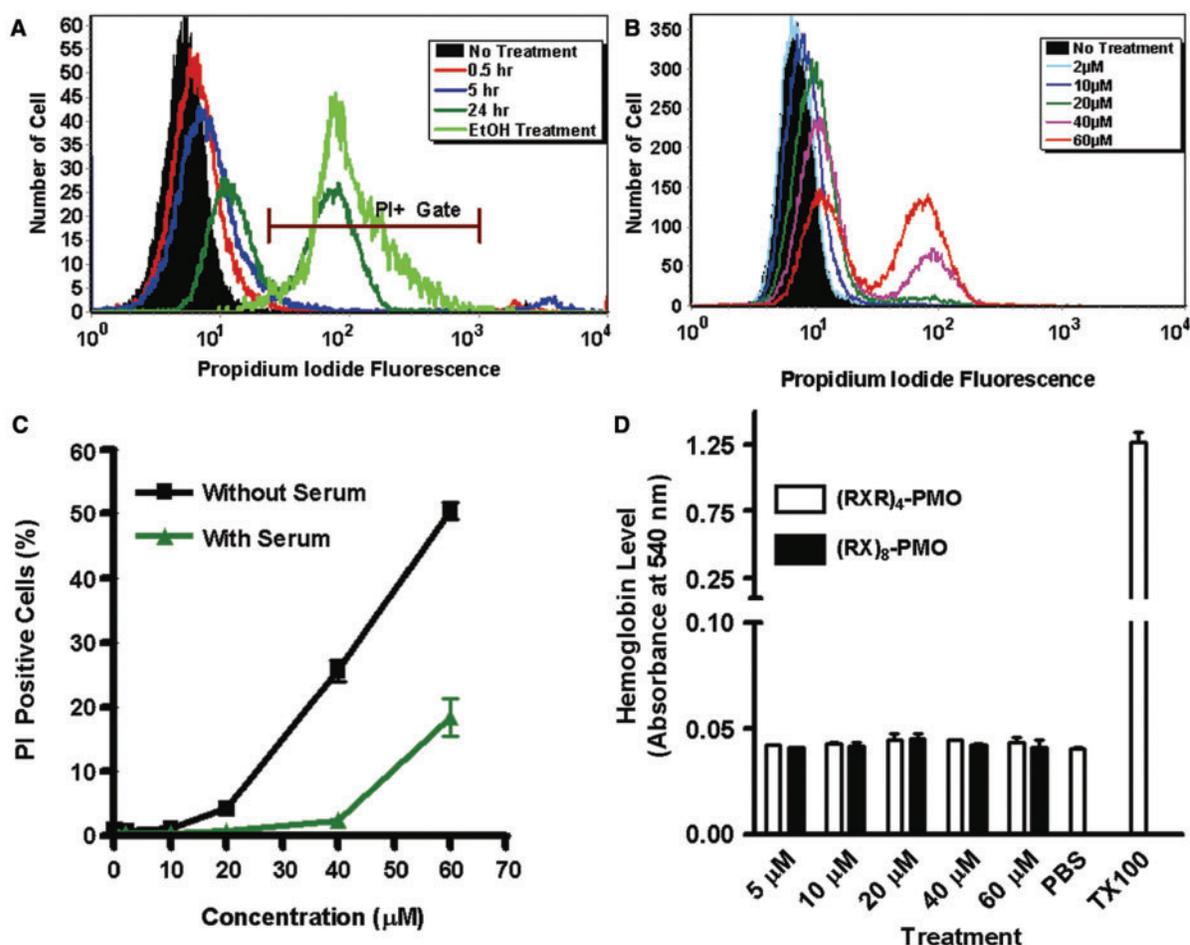
**Microscopic images.** We sought to verify the MTT results by collecting microscopic images of cells treated with 60  $\mu$ M of the conjugates. The images correlated well with the cell viability data. Images of  $(RX)_8$ -,  $(rX)_8$ -,  $(RXR)_3RBR$ - (4c),  $(RXR)_4$ -PMO and vehicle-treated cells are shown in Figure 7. Cells treated with  $(RX)_8$ -PMO and  $(RXR)_4$ -PMO appeared rounded and detached from the culture well, and appeared to have fewer live cells. Interestingly, cells treated with  $(rX)_8$ -PMO appeared to have normal morphology and cell density. The replacement of one X of  $(RXR)_4$ -PMO



**Figure 6.** Toxicity of **CPP-PMO** Conjugates: *MTT assay*. The cell viability is represented as the percent of viable pLuc 705 cells treated at 0, 2, 10, 20, 40 and 60  $\mu$ M of the indicated compounds. (A–E) in OptiMEM containing 10% serum for 24 h. (F) Cells were treated with arginine (500  $\mu$ M) and X (500  $\mu$ M) monomers independently and in combination (500  $\mu$ M R + 500  $\mu$ M X). Data are represented as a mean of six data points obtained from two independent experiments.



**Figure 7.** Toxicity of **CPP-PMO** conjugates: *microscopic images*. pLuc705 cells were treated with the vehicle (No treatment),  $(RX)_8$ -PMO,  $(rX)_8$ -PMO,  $(RXR)_4$ -PMO or  $(RXR)_3$ RBR-PMO (4c) at 60  $\mu$ M in OptiMEM containing 10% serum for 24 h and then directly visualized in the culture medium under a microscope at  $\times 100$  magnification.



**Figure 8.** Toxicity of **CPP-PMO** conjugates: *PI exclusion and hemolysis assay*. All treatments were carried out in OptiMEM in the presence and absence of 10% FBS. **(A)** Flow cytometry histograms of cell numbers versus PI fluorescence of HeLa cells treated with 60  $\mu\text{M}$  of  $(\text{RXR})_4\text{-PMO}$  for 0.5, 5, and 24 h in serum-free medium. Ethanol-treated cells are used as the positive control and the PI positive peak is indicated in the histogram. **(B)** Flow cytometry histograms of cells treated with  $(\text{RXR})_4\text{-PMO}$  at the concentration of 0, 2, 10, 20, 40 or 60  $\mu\text{M}$  for 24 h in serum-free medium. **(C)** Cells were treated with  $(\text{RXR})_4\text{-PMO}$  for 24 h in the absence and presence of 10% FBS and then subjected to PI treatment and the flow analysis. **(D)** The level of hemoglobin (absorbance at 540 nm) released from fresh rat red blood cells treated with  $(\text{RXR})_4\text{-PMO}$ ,  $(\text{RX})_8\text{-PMO}$ , PBS (negative control) or 0.005% of TX-100 (positive control) for 1 h at 37°C. The data represents a mean  $\pm$  SD of four data points from two independent experiments.

with one B reduced toxicity significantly;  $(\text{RXR})_3\text{RBR-PMO}$  ( $4\text{c-PMO}$ )- treated cells had similar density and morphology to the vehicle-treated cells.

**Propidium iodine exclusion assay.** The effect of the conjugates on integrity of cell membranes was investigated by a propidium iodine (PI) exclusion assay. PI can only permeate unhealthy/damaged membranes, so positive PI fluorescence indicates compromised cell membranes. Only  $(\text{RXR})_4\text{-}$  and  $(\text{RX})_8\text{-PMO}$  conjugates were found to significantly affect membrane integrity at higher concentrations (up to 60  $\mu\text{M}$  tested). Figure 8A shows the histograms of pLuc705 cells treated with  $(\text{RXR})_4\text{-PMO}$  at 60  $\mu\text{M}$  for 0.5, 5 and 24 h. The PI positive (PI+) region was defined by the cells permeabilized with ethanol (positive control) as indicated by the gate in the histogram. The PI histogram shifts from the PI-negative region to PI-positive region in the longer incubations, indicating the conjugate caused membrane leakage in a time-dependent manner. The 0.5- and 5-h-treatments

caused a slight shift towards the PI+ region, while the 24-h-treatment produced a distinct peak which corresponded to 57% of cells that were in the PI+ region. Figure 8B shows the histograms of cells treated with  $(\text{RXR})_4\text{-PMO}$  at concentrations of 2, 10, 20, 40 and 60  $\mu\text{M}$  for 24 h. There was no significant PI uptake at concentrations up to 20  $\mu\text{M}$ . At higher concentrations, the PI+ population appeared and the percentage of PI+ cells increased as the treatment concentration increased, indicating that there were more leaking cells at the higher treatment concentration. Similar concentration- and time-dependent PI uptake profiles were observed for  $(\text{RX})_8\text{-PMO}$  but not for  $(\text{RB})_8\text{-PMO}$  and the remaining conjugates (data not shown). Addition of 10% serum to the treatment medium significantly reduced membrane toxicity for the  $(\text{RXR})_4\text{-}$  (Figure 8C) and  $(\text{RX})_8\text{-PMO}$  conjugates (data not shown).

**Hemolysis assay.** The  $(\text{RXR})_4\text{-}$  and  $(\text{RX})_8\text{-PMO}$  conjugates were tested in a hemolysis assay and found to be

compatible with red blood cells. Fresh rat red blood cells were treated with the conjugates at 60  $\mu$ M, PBS (background) or 0.005% TX-100 (positive control). The supernatants of conjugate- and PBS-treated samples had small and similar amounts of free hemoglobin released, far lower than that of the TX-100-treated samples (Figure 8D).

## DISCUSSION

The naturally occurring CPPs such as Tat peptide are not stable in blood and neither are oligolysine/oligoarginine (5), rendering these CPPs unfavorable as transporters for therapeutic AOs. We reasoned that one approach to improve stability would be to use non- $\alpha$  amino acids or D-amino acids. In this study, we investigated whether incorporation of 6-aminohexanoic acid (X),  $\beta$ -alanine (B) and D-arginine (r) amino acids into the CPP would affect cellular delivery, antisense activity, toxicity and serum binding of the resulting CPP-PMO conjugates.

We found that CPP-PMOF conjugates containing X/B residues did not enter cells as efficiently as R<sub>8</sub>- and R<sub>9</sub>-PMO conjugates. This is consistent with our previous finding for the (RXR)<sub>4</sub> conjugate (6). We have found that cell surface proteoglycans were involved with binding of the Tat-, R<sub>9</sub>F<sub>2</sub>- and (RXR)<sub>4</sub>-PMO conjugates with the (RXR)<sub>4</sub> conjugate having the lowest binding affinity. Insertion of X into an oligoarginine CPP reduces the charge density and may lead to decreased binding affinity for proteoglycans. Despite the lower cellular uptake of X/B-containing CPP-PMO, they generated higher antisense activities in the cell nucleus than oligoarginine-PMO. We have found that endocytosis was the internalization mechanism (at least primarily) for oligoarginine- and (RXR)<sub>4</sub>-PMO conjugates. Indication of different uptake mechanisms was not found among these conjugates (6). Therefore we hypothesize that X/B-containing conjugates have a greater ability to escape from endosomes/lysosomes than oligoarginine conjugates by a mechanism as yet to be studied.

The number of X residues affects both the nuclear antisense activity and the toxicity of conjugates. The CPP-PMO conjugate with 8 X residues [(RX)<sub>8</sub>-PMO] had the highest activity followed by one with 5 Xs [(RXR)<sub>4</sub>-PMO] (Figures 1 and 4). However, these conjugates were toxic to cells at higher concentrations, which may be a concern when considering potential applications for *in vivo* delivery of PMO. Replacement of all 8 Xs with Bs decreased both toxicity and antisense activity. The combination of 3-4 Xs with several B residues yielded CPPs with no detectable toxicity, and at some concentrations several of them had similar antisense activity as (RX)<sub>8</sub>-PMO. We think this type of CPP, having Bs and fewer than 5 Xs, will offer balanced activity and low toxicity as well as the stability, and have considerable potential for delivery of therapeutic AOs. Further investigation into the toxicity and activity versus dosing levels of these CPPs *in vivo* is warranted.

Surprisingly, the replacement of L-arginine with D-arginine enhanced neither uptake nor antisense activity for oligoarginine, or X- and B-containing conjugates. In the case of (RX)<sub>8</sub>-PMO, the replacement actually caused a small but statistically significant decrease in activity. Our observation is different from the results reported by others (20,21) who found that D-CPPs had higher cellular uptake than L-CPPs, although no biological functional cargo was used in their study. The difference between results may be due to the type and size of cargos and the cell lines used for the assays. Whether the use of D-arginine-containing peptides results in superior CPP-PMO functional activity *in vivo* remains to be tested.

We attempted to understand the nature of (RX)<sub>8</sub>- and (RXR)<sub>4</sub>-PMO toxicity. It is apparent that these two conjugates caused little immediate membrane damage with 0.5 or 5 h treatment at concentrations as high as 60  $\mu$ M (Figure 8). However, these two conjugates had dose-dependent toxicity with 24 hr treatment as shown by the leaky cell membranes and fewer cells compared to controls (Figure 6C&D, Figure 8). Interestingly, the replacement of Xs with Bs in (RX)<sub>8</sub>-PMO abolished the toxicity, and the replacement of L-arginine with D-arginine reduced the toxicity of (RX)<sub>8</sub>-PMO (Figure 6). We have found that (rX)<sub>8</sub>-PMO was completely stable and the peptide portion of (RB)<sub>8</sub>-PMO was only partially degraded, whereas the peptide portion of (RX)<sub>8</sub>-PMO was completely degraded in cells (5). We wondered whether the difference in toxicity among (RX)<sub>8</sub>, (RB)<sub>8</sub> and (rX)<sub>8</sub>-PMO conjugates was caused by differences in intracellular stability, resulting in the metabolized products of (RX)<sub>8</sub>-PMO producing toxicity. The identifiable metabolized products of (RX)<sub>8</sub>-PMO were XRXB-PMO and XB-PMO (5) but neither product had any detectable toxicity as measured by MTT assay (data not shown). It is possible that the CPP portion was degraded into free amino acids and/or smaller peptide fragments which were toxic. However, our investigation revealed that neither free R nor X, alone or in combination, caused cellular toxicity. Another possibility is that because of the high hydrophobicity of X compared to B, X in combination with positively charged arginine residues leads to toxicity not generated by B residue combinations. However, this explanation does not account for the difference in toxicity observed between (RX)<sub>8</sub>-PMO and (rX)<sub>8</sub>-PMO, which have the same hydrophobicity. Perhaps the toxicity of (RXR)<sub>4</sub>-PMO and (RX)<sub>8</sub>-PMO was caused by the peptide fragments that we could not identify by mass spectrometry.

Unlike the toxicity difference between (RX)<sub>8</sub>- and (rX)<sub>8</sub>-PMO, the L $\rightarrow$ D replacement did not change the toxicity of (RXR)<sub>4</sub>-PMO (Figure 6). Substitution of either one R (rXR) or two R (rXr) from the RXR repeat neither reduced nor increased the toxicity profile of (RXR)<sub>4</sub>-PMO. At this point, we do not fully understand the mechanisms of (RXR)<sub>4</sub>- and (RX)<sub>8</sub>-PMO conjugate toxicity, but look forward to studying this topic further.

Serum effect on the activity of a CPP-AO conjugates is an important issue when considering potential *in vivo* applications. X/B-containing conjugates were still active in 60% serum while oligoarginine conjugates were not.

The greater stability of the X/B-containing conjugates to serum enzymes is likely a factor contributing to their high activity. The loss of activity in high serum concentrations makes oligoarginine CPPs undesirable as potential therapeutic AO carriers.

In summary, we have found that the X/B-containing **CPP-PMO** conjugates are superior to oligoarginine-PMO conjugates for the following reasons: they display higher activity in cell nuclei, are less affected by serum and are more stable in blood (5). The toxicity of the X/B-containing CPPs can be reduced by keeping the number of X residues below 5 while still maintaining a reasonable delivery efficacy and stability. This study provides a basis for further optimization of CPP sequence using R, r, X and B residues in the interest of further reducing toxicity and increasing antisense activity, which will likely lead to more effective AO transporters for potential therapeutic applications.

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