Chapter 4

Synthesis and toxicology studies of a Py-Im polyamide targeted to the CTG•CAG-repeat sequence of Myotonic Dystrophy

This research was conducted in collaboration with Alissa A. Hare, Alexis A. Kurmis, Jordan L. Meier, Fei Yang, and Peter B. Dervan (California Institute of Technology) and with Joel Gottesfeld and Jintang Du (Scripps Research Institute).

Abstract

Myotonic dystrophy type 1 (DM1) is a neuromuscular disease caused by an expansion of CTG•CAG triplet repeats which, when transcribed into mRNA, sequesters the RNA splicing regulator muscleblind-like protein from its normal function and causes clinical pathology. DNA binding Py-Im polyamides targeted to the CTG•CAG triplet repeat sequence may be useful towards disrupting transcription of the toxic RNA. In this study, we assess the toxicity of two polyamides in mice to inform tolerable dosing regimens to be examined in a DM1 mouse model. We found that subcutaneously injected hairpin polyamide **2** targeted to 5'-(A/T)GC(A/T)GC(A/T)-3' is absorbed and circulates in a fashion similar to previously studied eight-ring polyamides. However, polyamide **2** dosed three times a week displayed toxicity at low (1 mg/kg) doses and serum chemistry showed liver and kidney damage at a single dose of 5 and 10 mg/kg. Polyamide **3**, which differs only by an acetylation at the butyric acid turn unit, showed no toxicity at a single dose of up to 9 mg/kg but was dose limited by solubility. This study suggests polyamide **3** is a better tolerated compound and should be assessed for circulation and multiple dose toxicity, but its formulation should be improved.

4.1 Introduction

Myotonic dystrophy type 1 (DM1) is an inherited neuromuscular disorder with a wide-spectrum of clinical pathologies which include dysfunction of skeletal muscles, cardiac defects, and cataracts.[1] The disease is caused by a pathological expansion of CTG•CAG triplet repeats at the 3' untranslated region of the gene encoding serine-threonine protein kinase DMPK1.[2] The expanded triplet repeats are transcribed into mRNA, which sequesters the RNA splicing regulator muscleblind-like 1 protein (MBNL1) to form nuclear foci.[3] MBNL1 governs exon splicing in a selection of genes and its sequestration results in splicing defects that cause clinical toxicity (Figure 4.1).[4]

Disease models and phenotypic assays have been developed to discover means to disrupt this pathology. In cell culture, potential therapeutic strategies can be screened for reduction in the number of toxic nuclear foci. For screening in animals, Thornton and coworkers have developed a mouse model with muscle-specific expression of skeletal

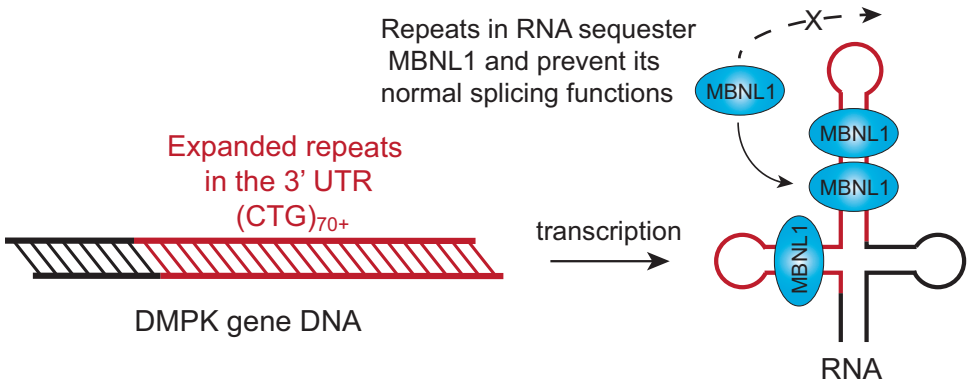


Figure 4.1 Diagram of the molecular cause of Myotonic Dystrophy type 1. Pathogenic RNA transcribed from expanded CTG•CAG triplet repeats binds MBNL1. MBNL1 is not available to perform its normal RNA splicing functions and results in toxicity.

actin containing the expanded triplet repeats.[5] The model successfully recapitulated sequestration of MBNL1, splicing defects, and myotonia. This model has been used to show that RNA interference technology can prevent sequestration of MBNL1 and reverse pathology.[6] RNA interference is a reasonable method to degrade the triplet-repeat expanded transcripts after their production, but its therapeutic utility is challenged by poor organismal distribution.[7] An alternative approach to reducing toxic mRNA is to decrease the transcription of the expanded repeat sequence, which potentially can be accomplished by sequence-specific DNA-binding small molecules.

Py-Im polyamides are a class of programmable, sequence-specific small molecules that bind in the minor groove of DNA.[8-13] Sequence preference is achieved by side-by-side pairings of aromatic amino acids that distinguish the edges of the four Watson-Crick base pairs according to the pairing rules: Im/Py codes for a G•C base pair and Py/Py binds both T•A/A•T in preference to G•C/C•G.[11-14] Eight-ring hairpin polyamides linked by a central aliphatic γ-aminobutyric acid unit have affinities for match sites with K_a's from 10^8 to 10^{10} M^{-1}.[9,15] Targeting longer sequences in this hairpin motif is challenging because the oligomers are over-curved compared to DNA.[16,17] To relax the curvature of oligomers targeting longer sequences, β alanine (β) can be substituted for Py-rings in some cases such that β/β pairs replace Py/Py for T•A/A•T specificity.[16-18] Access to chromatin without transfection agents has been demonstrated by fluorescent dye conjugates of cell-permeable polyamides which localize to the nucleus in live cells.[19,20] Hairpin polyamides have been shown to downregulate transcription of

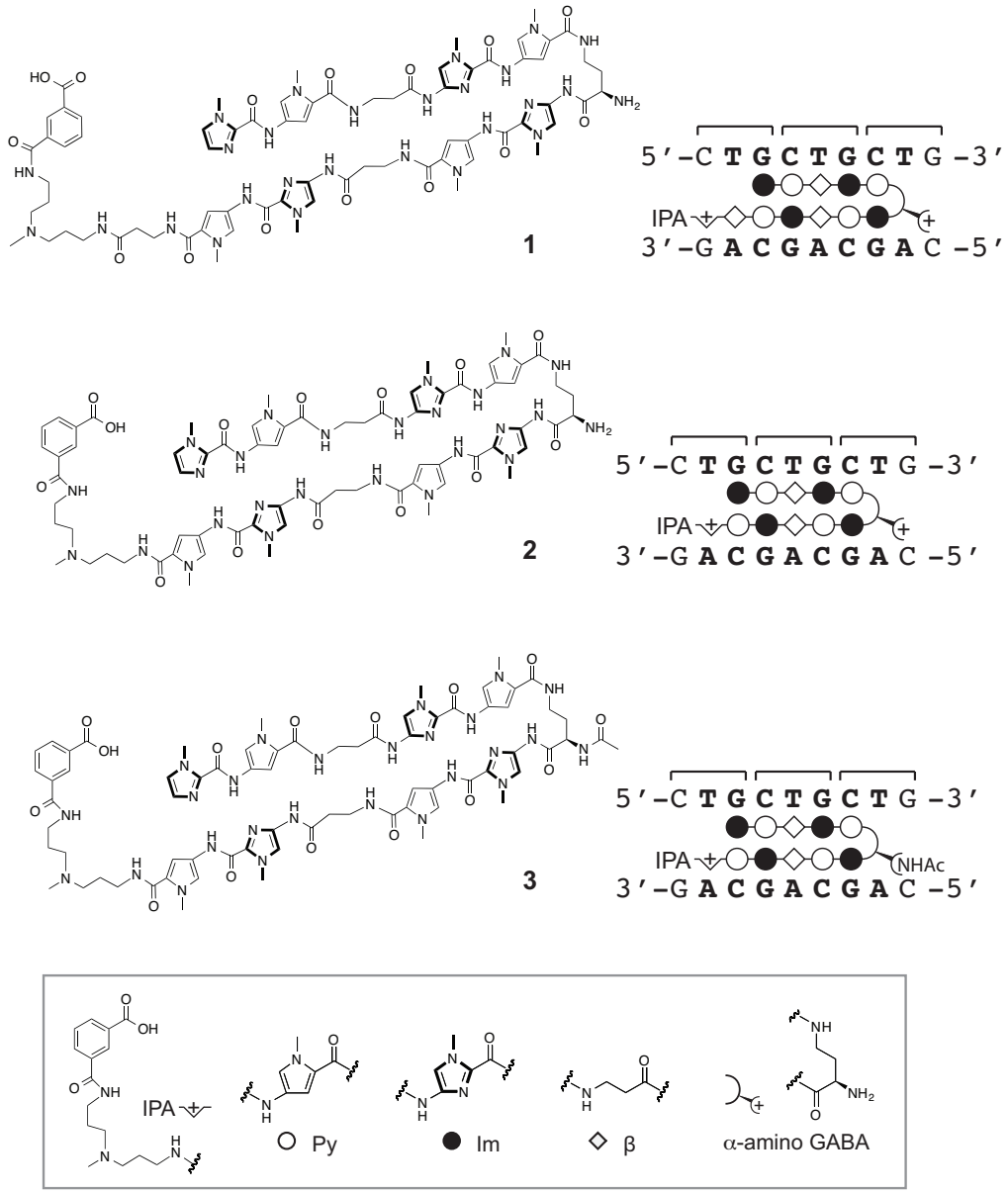
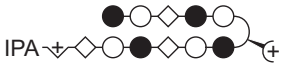
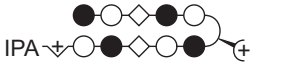
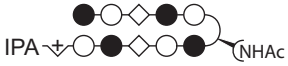


Figure 4.2 Structures of polyamides synthesized for this study. Polyamide **1-3** contain the same core and target the same 5'-(A/T)GC(A/T)GC(A/T)-3' sequence contained in the triplet repeat sequence but differ at the C-terminal tail and the hairpin turn unit.

associated genes when targeted to the consensus sequences of binding sites of transcription factors in both cells and xenograft models in mice.[21-25] A Py-Im polyamide targeted to the CTG•CAG repeat sequence may downregulate the transcription of the

Table 4.1 Thermal stabilization of DNA by 2β2 polyamides.

5′–A C **T G C T G C T** G A T –3′
3′–T G **A C G A C G A** C T A –5′

		T_m ºC	ΔT_m ºC
DNA only		57.0 (±0.3)	- -
	**1**	73.7 (±0.2)	16.7
	**2**	76.2 (±0.1)	19.1
	**3**	71.2 (±0.2)	14.2

DMPK1 gene which causes the DM1 pathology. Studies in mouse models have shown Py-Im polyamides circulate with good distribution and pharmacokinetic profiles,[26,27] such that Py-Im oligomers may provide an avenue for therapeutic benefit.

4.2 Results

Py-Im polyamides **1**, **2**, and **3** are hairpin polyamides with 2 aromatic rings β–alanine and 2 aromatic rings on each strand (2β2), designed and synthesized to target the CTG•CAG repeat sequence (Figure 4.2). Their DNA binding to the CTG repeat sequence 5'-AC**TGCTGCT**GAT-3' was verified by a DNA thermal denaturation experiment, which correlates with binding affinity. [28] The results show robust stabilization of the duplex DNA by all three polyamides, with ΔT_m values of 16.7 ºC, 19.1 ºC, and 14.2 ºC for 2β2 hairpins **1**, **2**, and **3**, respectively (Table 4.1). In on-going studies, these compounds are being evaluated by our collaborators in the Gottesfeld group in a cell culture model of DM1. Induced pluripotent stem cells (iPSC) with

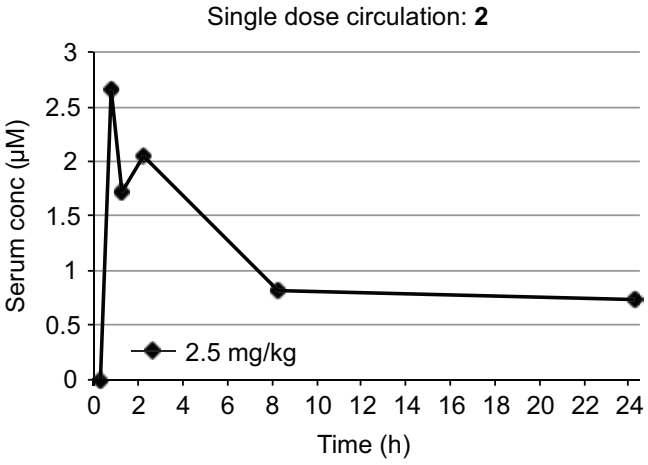


Figure 4.3 Circulation study of polyamide **2** in normal C57BL/6 male mice. Single subcutaneous dose in 20% DMSO/PBS given to four mice, two mice per time point. Concentrations were obtained by analytical HPLC against an internal standard.

CTG•CAG expanded repeats were produced using transcription factor reprogramming of DM1 patient fibroblasts. As their preliminary results suggested a reduction in nuclear RNA foci, we began studies in parallel to assess the toxicity of the compounds in normal mice. The aim of this study is to assess toxicity to inform tolerable dosing regimens to be examined for efficacy in the expanded-repeat DM1 mouse model.

We first chose to assess hairpin polyamide **2** in mice with the consideration that the C-terminal tail structure without the β–alanine has been better characterized in both live cell and mouse studies, and its solubility properties were better than that of **3.** To confirm circulation of the hairpin polyamide, 4 normal C57BL/6 mice were injected subcutaneously with polyamide **2** at a concentration 2.5 mg/kg. We applied a single dose at 200 μl and were limited by the solubility of the compound in the vehicle, 20% DMSO in PBS. Nevertheless, we detected the compound in the blood stream within half an hour of injection at 2.7 μM (Figure 4.3). The dip in concentration at 1 hour is likely variability

between mice because time points are alternated between groups of two mice each; this would likely average out with more replicates. At 8 hours and 24 hours, the detected peaks are near the lower detection limit for the HPLC. In proportion to the amount of polyamide **2** injected, the concentration and time course of elimination is similar to that previously observed with subcutaneously injected eight-ring hairpins.[26]

To achieve higher doses in a single dose experiment of toxicity, we exchanged PBS for saline in the vehicle, which allowed us to achieve concentrations of 5 mg/kg per 200 µl injection. On day 0, we injected male C57BL/6 mice subcutaneously with one or two injections to achieve doses of 5 mg/kg (n = 3) and 10 mg/kg (n = 1), respectively, and observed their weight over 9 days (Figure 4.4A and 4.4B). By day 6, the mouse dosed at 10 mg/kg had lost over 15% of its weight and had to be euthanized. At day 9, the end of the study, mice dosed at 5 mg/kg were distressed and averaged 87% of their initial weight (n=3). Blood serum was obtained from the mice at the end of the study or upon sacrifice for blood chemistry analysis. We found a large elevation of aspartate transaminase (AST), alanine transaminase (ALT), and total bilirubin in the blood, indicative of liver toxicity in a dose dependent manner (Table 4.2). Blood urea nitrogen (BUN) and creatinine are also elevated, suggesting the mice sustained kidney damage as well (Table 4.2). This showed the maximum tolerated single dose is under 5 mg/kg for polyamide **2**.

We next explored lower doses in anticipation of multiple dose studies. At 1 mg/kg (n = 1) and 2 mg/kg (n = 1), there was no decrease in weight observed over the 9 days

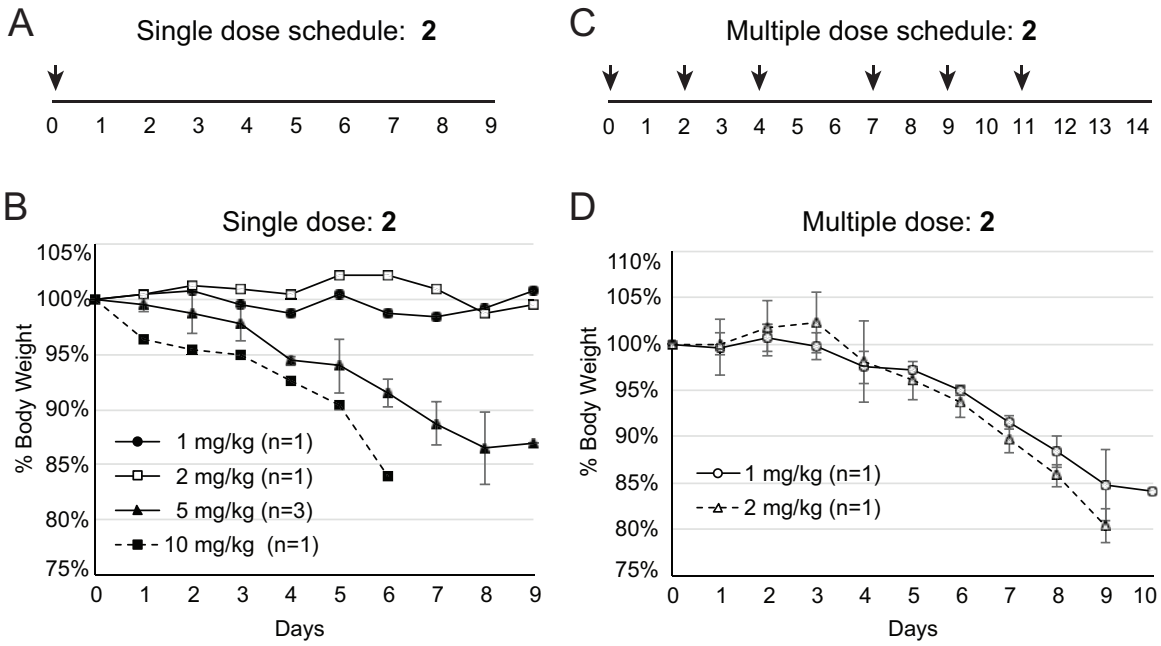


Figure 4.4 Weight curve studies with polyamide **2**. A) Schedule of dosing and observation for single dose study with polyamide **2**. B) Weight curves for escalating single doses: 1, 2, 5, and 10 mg/kg dosed subcutaneously in 20% DMSO/saline. C) Schedule of dosing and observation for multiple dose study with polyamide **2**. D) Weight curves for multiple doses at 1 or 2 mg/kg dosed subcutaneously. Mice were euthanized prior to end of study due to weight loss greater than 15%.

following injection (Figure 4.4B). We then assessed the tolerability of hairpin **2** after multiple dose exposure at these concentrations. The study was designed for three injections per week, for two weeks (Figure 4.4C). Mice were injected at 1 mg/kg (n = 2) and 2 mg/kg (n = 2) and their weights monitored. Mice injected at 2 mg/kg lost more than 15% of their weight by day 9 and were euthanized. Mice injected at 1 mg/kg were euthanized after losing more than 15% of their body weight by day 10, such that no mice survived the experiment (Figure 4.4D).

Given the significant toxicity observed with polyamide **2**, we tested polyamide **3**, as previous studies have indicated acetylation of the amine on the γ-butyric acid turn unit

Table 4.2 Serum chemistry analysis for liver and kidney toxicity.

Polyamide	ALT	AST	Bilirubin	BUN	Creatinine
Normal	57 ±40	194 ±152	0.32 ±0.3	23.6 ±4.8	0.34 ±0.12
Vehicle	17	45	0.1	30	0.2
1 5 mg/kg	1343	798	0.3	50	0.7
1 10 mg/kg	1944	1297	0.2	79	0.9

can improve the toxicity profile.[29] Polyamide **3** was dissolved in 20% DMSO in saline, but was only soluble to 2.5 mg/kg. Doses of 9 mg/kg (n = 1) and 5 mg/kg (n = 1) were achieved with 4 and 2 injections, respectively. Despite the large volume of vehicle injected into the mice, both doses showed no toxic effect on the mice, and the mice gained weight through the 9 days of observation (Figure 4.5A and B). These results suggest the 2β2 hairpins **2** and **3** follow the same pattern of reduced toxicity shown for eight-ring hairpins upon acetylation of the amine on the turn unit.

4.3 Discussion

From these studies we have found that the 2β2 hairpin polyamide **2** circulates in a fashion similar to eight ring polyamides dosed subcutaneously. However, we found that polyamide **2** is toxic at a single dose of 5 mg/kg and at 1 mg/kg dosed three times a week. Indeed, a single injection at 5 and 10 mg/kg caused liver and kidney damage. This compound is likely too toxic for long term dosing in the DM1 mouse model. Due to this toxicity, the acetylated 2β2 hairpin **3** was examined. Polyamide **3** showed a more

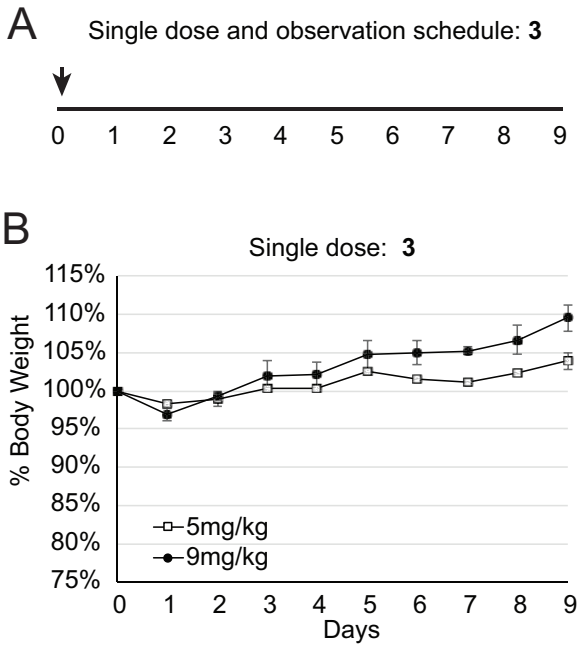


Figure 4.5 Weight curve studies with polyamide **3**. A) Schedule of dosing and observation for single dose study with polyamide **3**. B) Weight curves for single doses delivered in 2 or 4 subcutaneous injections of 20% DMSO/saline.

favorable tolerability profile by weight assessment after a single dose at 5 and 9 mg/kg, dose levels that caused toxicity in with polyamide **2**. The circulation of this compound and its tolerability after multiple doses remains to be tested.

The insolubility of the acetylated 2β2 oligomer **3** in the 20% DMSO/saline vehicle will limit achievable compound exposure in the mouse model and motivates exploration of improved formulation. While dose titration will be an iterative process with the efficacy model, an improved formulation will be advantageous for testing higher concentrations. It remains to be studied whether an alteration in formulation will substantively change the pharmacokinetic and toxicity profiles of the polyamide. The

establishment of a well-tolerated dosing window in mice will be the first step towards assessing the utility of 2β2 hairpin polyamides for addressing DM1 pathology.

4.4 Materials and Methods

Synthesis of polyamides. Hairpin polyamide **1** was synthesized on β-alanine Pam-resin and polyamides **2** and **3** were synthesized on Kaiser oxime resin as previously described.[30-32] Polyamides were purified by preparatory HPLC and the purity and identity determined by analytical HPLC and MALDI-TOF (Table 4.3).

Thermal denaturation studies. The DNA oligomer 5'-ACTGCTGCTGAT-3' and its complement were purchased from Integrated DNA Technology and annealed to form duplex DNA. DNA and each hairpin polyamide was mixed to a final concentration of 2 and 3 μM, respectively, in 1 mL total volume. The buffer was an aqueous solution of 10 mM sodium cacodylate, 10 mM KCl, 10 mM $MgCl_2$, and 5 mM $CaCl_2$ at pH 7. A Varian Cary 100 spectrophotometer with a thermocontroller was used for measurements at λ = 260 nm with a path length of 1 cm. Samples were heated to 90 °C and cooled to a starting temperature of 25 °C and then heated at a rate of 0.5 °C/min to 90 °C to record denaturation profiles. Melting temperature was defined as the maximum of the first derivative of the denaturation profile. Data is the mean of four measurements.

Mouse studies. All animal experiments were conducted under an IACUC approved protocol at the California Institute of Technology. 6-8 week old male C57BL/6J mice were purchased from Jackson Laboratory and allowed to acclimate for at least 3 days.

Table 4.3 Masses of polyamides by MALDI-TOF.

Polyamide	Formula	[Mass + H]	Found Mass
1	$C_{72}H_{90}N_{27}O_{15}^+$	1572.7	1572.7
2	$C_{69}H_{85}N_{26}O_{14}^+$	1501.7	1501.6
3	$C_{71}H_{87}N_{26}O_{15}^+$	1543.7	1543.7

Circulation of compound 2. The circulation experiment was conducted as previously described for 8-ring hairpin polyamide analyses. [26] Four acclimatized 8-week old male C57BL/6J mice were separated into two cages and all dosed with polyamide **2** in a subcutaneous single injection in the flank. Solubility limited the concentration to 2.5 mg/kg dosed in 200 µl of 20% DMSO/PBS. Blood was obtained by retro-orbital collection at 0.5, 1, 2, 8, and 24 hours from alternating pairs of mice. The blood from two mice was combined and serum isolated by centrifugation at 6000 × g for 5 minutes. 40 µl of serum was combined with 80 µl of MeOH and vortexed to precipitate proteins. The samples were centrifuged at 16000 × g and 90 µl of the supernatant removed to a new tube. An equal volume of 20% acetonitrile in 0.1% TFA/water was added. 20 µl of an internal standard, Boc-Im-OEt, (~ 1 mg in 10 ml water) water was spiked into 180 µl of sample.

The samples were analyzed on a Beckman Gold analytical HPLC with a Phenomenex Kinetex C18 analytical column (100 mm × 4.6 mm, 2.6 µm). Peaks for polyamide **2** were quantitated by integration at 304 nm relative to the internal standard and determined by interpolation on a standard curve. A standard curve of concentration versus integrated area was generated by diluting known concentrations of polyamide **2** into C57BL/6 serum and preparing the samples with the internal standard in the same manner as above.

Single dose or multiple dose animal weight loss. Animals were injected at the described doses and schedules with U-100 insulin syringes (UltiCare). Treatment was given subcutaneously in 20% DMSO in saline or PBS as vehicle. Animals were monitored daily for weight for 9 or 14 days (single dose or multiple dose, respectively) and sacrificed if weight loss was greater than 15%.

Serum chemistry analysis. Serum analysis was conducted as previously described. [29] In brief, blood from treated mice was obtained by retro-orbital bleeding (Drummond) and serum isolated after centrifugation at 6000 × g for 5 minutes. Serum ALT, AST, total bilirubin, BUN, and creatinine levels was analyzed at IDEXX-RADIL. Normal values are reported from The Jackson Laboratory (http://jaxmice.jax.org/strain/000664.html).

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