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Label-free fluorescence detection of the depurination activity of ribosome inactivating protein toxins

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Electronic Supplementary Information

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1. Materials: Custom synthesized oligoribonucleotides **1** and **3** were purchased from Dharmacon RNAi Technologies. Oligoribonucleotides were deprotected according to the supplier's protocol, purified by gel electrophoresis under denaturing conditions and desalted using Sep-Pak Classic C18 cartridges (Waters Corporation). DNA oligonucleotide **4** was purchased from Integrated DNA Technologies, Inc. Saporin was purchased from Sigma-Aldrich. ATMND was purchased from Enamine Ltd., Ukraine**.** Chemicals for preparing buffer solutions were purchased from Sigma-Aldrich (BioUltra grade). All oligonucleotide stocks and buffer solutions for saporin-mediated reactions and fluorescence measurements were prepared in autoclaved water.

2. Instrumentation: Absorption spectra were recorded on a PerkinElmer, Lambda 45 UV-vis Spectrophotometer. UV-thermal melting studies of oligonucleotides were performed on a Cary 300Bio UV-vis spectrophotometer. Steady-State fluorescence experiments were carried out in a micro fluorescence cuvette (Hellma, path length 1.0 cm) on a TCSPC instrument (Horiba Jobin Yvon, Fluorolog-3).

3. Optimization of titration conditions: It has been reported that the depurination reaction by saporin is significantly site-specific around pH 6.0.[S1] Also, saporin-mediated depurination reactions of short RNA substrates have been performed in Tris-HCl at pH 6.0. However, ATMND titrations have been mostly performed in cacodylate buffer at pH 7.0.[S2] In order to optimize the conditions to detect abasic sites, fluorescence titrations have been carried out using model systems in cacodylate and Tris-HCl buffers.

Model abasic site containing duplex **3**·**4** was assembled by heating a 1:1 mixture of the oligonucleotides **3** and **4** (final 10 μM) in 20 mM cacodylate buffer (pH 7.0, 100 mM NaCl, 0.5 mM EDTA) at 90 °C for 3 min and cooling the solution slowly to room temperature. The duplex stock solution was placed on a crushed ice bath for 60 min before use. To a solution of ATMND (200 μL, 100 nM) in 20 mM cacodylate buffer (100 mM NaCl, 0.5 mM EDTA, pH 7.0) was added aliquots of duplex **3**·**4**. The sample after each addition was excited at 350 nm and the change in ATMND fluorescence was monitored by measuring the emission at λ_{em}=406 nm (18 °C). The change in volume over the entire titration was ≤7%. Similarly, titrations were also performed in 30 mM Tris-HCl buffer (25 mM NaCl, 2 mM $MgCl_2$, pH 7.0). Titration of ATMND with the model abasic site containing duplex **3**·**4** in cacodylate buffer (pH 6.0 and 7.0) shows drastic quenching in

fluorescence intensity (nearly 4-fold, Fig. S1). Titration of ATMND with duplex **3**·**4** in Tris-HCl buffer at pH 6.0 and 7.0 shows a 3-fold quenching in fluorescence intensity (Fig. S1).

Next, ATMND was titrated with model duplex **3**·**4** in Tris-cacodylate buffer combination, with the view of performing the depurination reaction in Tris-HCl buffer (pH 6.0) and hybridization with complementary DNA oligonucleotide **4** using cacodylate buffer (pH 8.0). The following ATMND stock and buffer solutions were prepared.

ATMND stock: 200 nM in 20 mM cacodylate buffer (100 mM NaCl, 0.5 mM EDTA, pH 8.0)
Hybridization buffer: 20 mM cacodylate buffer (175 mM NaCl, 0.5 mM EDTA, pH 8.0)
Reaction buffer: 30 mM Tris-HCl (25 mM NaCl, 2 mM $MgCl_2$, pH 6.0)

ATMND (200 µL, 100 nM) was prepared by mixing 100 µL of ATMND stock solution, 50 µL of hybridization buffer and 50 µL of reaction buffer. This Tris-cacodylate buffer combination gave a final pH of 7.2. To the above ATMND solution was added aliquots of duplex **3**·**4** (10 µM), and changes in ATMND fluorescence were measured as before. In this buffer combination more than 5-fold quenching of ATMND fluorescence was observed, and the extent of quenching was better compared to titrations in cacodylate and Tris-HCl buffers alone (see Fig. 2). Therefore, fluorescence detection of saporin-mediated depurination of RNA substrate **1** was performed in Tris-cacodylate buffer combination.

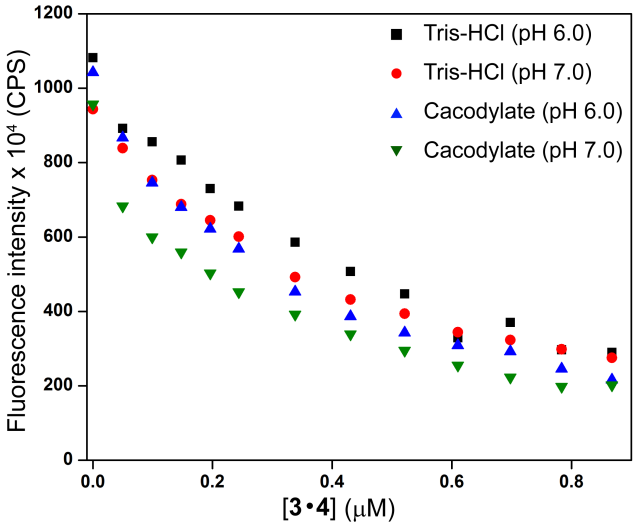

Fig. S1 Fluorescence titration of ATMND with an abasic site containing model duplex **3**·**4** in Tris-HCl and cacodylate buffers. Samples were excited at 350 nm with excitation and emission slit widths of 6 and 9 nm, respectively. The fluorescence of ATMND was measured at λ_{em}=406 nm.

4. Binding affinity: The binding constant (K_{11}) for the complexation of ATMND to the duplex **3·4** was estimated by analyzing the fluorescence titration curves using a nonlinear least-squares regression based on a 1:1 binding isotherm model.[S3] Igor Pro 6.0 software was used for curve fitting. This binding isotherm model has been previously used in the determination of K_{11} for the complexation of ATMND to various abasic site-containing DNA duplexes.[S3]

$$F/F_0 = \{1 + kK_{11}[D]\}/\{1 + K_{11}[D]\}$$

F and F_0 are the fluorescence intensities of ATMND in the presence and absence of the duplex **3·4**, respectively; $k = k_{11}/k_L$ represents the ratio of proportionality constants connecting the fluorescence intensities and concentrations of the species (1:1 complex, k_{11}; free ligand, k_L). The free duplex concentration [D] can be related to known total concentrations of duplex [D_0] and ligand [L_0] by the following equation.

$$D_0 = [D] + \{L_0K_{11}[D]\}/\{1 + K_{11}[D]\}$$

Together, the above two equations describe the system.

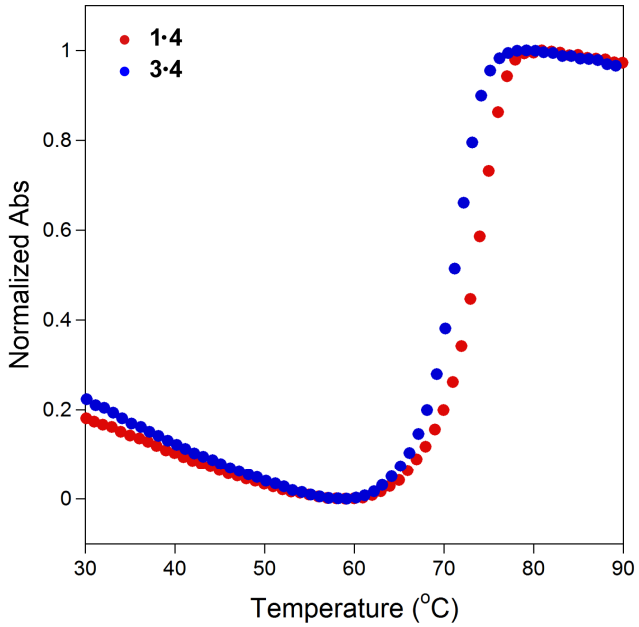

Fig. S2 UV-thermal melting analysis of duplexes **1·4** and **3·4** (1 μM) in 20 mM cacodylate buffer (pH 7.0, 100 mM NaCl, 0.5 mM EDTA). Duplexes were formed by heating a 1:1 mixture of oligonucleotides at 90 °C for 3 min, and cooling the solutions slowly to room temperature. Samples were placed in crushed ice for at least 60 min before analysis. T_m for duplexes **1·4** and **3·4** are 74.5 ± 0.6 °C and 71.5 ± 0.9 °C, respectively.

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5. Fluorescence detection of the depurination activity of saporin: A solution of RIP oligoribonucleotide substrate **1** (2 μM) in Tris-HCl reaction buffer (see section 3) was hybridized into a stem-loop structure by heating the sample at 75 °C for 3 min and slowly cooling it to RT. The sample was placed on crushed ice for 1 h and later incubated at 37 °C for 10 min. Depurination reactions were initiated by adding saporin (4.00, 2.00, 0.50 and 0.25 μM) at 37 °C. Aliquots (50 μL) of reaction mixtures were mixed with complementary DNA oligonucleotide **4** (50 μL, 2.2 μM in hybridization buffer, see above) and was immediately hybridized by heating at 90 °C for 3 min and flash cooling on crushed ice. Hybridized samples (100 μL) were then mixed with 100 μL of ATMND stock (200 nM in 20 mM cacodylate buffer, 100 mM NaCl, 0.5 mM EDTA, pH 8.0) and centrifuged. The samples were then equilibrated at 18 °C for 4 min and were excited at 350 nm with an excitation and emission slit width of 6 and 9 nm, respectively. The depurination activity was monitored by measuring the quenching of ATMND fluorescence intensity at λ_{em}=406 nm. All reactions were performed in triplicate. The rate of decrease of ATMND fluorescence is directly proportional to the rate of formation of the abasic RNA product **2**. For Figure 3, the concentration of duplex **2·4** was estimated from a saporin (0.5 μM) mediated depurination reaction of substrate **1** using above relationship.

6. Rate of depurination reaction: Fluorescence intensity at a given reaction time point corresponds to the sum of the fluorescence of free ATMND ligand and ligand-product duplex (**2·4)** complex (equation 1). The rate of depurination reaction is proportional to the formation of the ligand-product duplex (**2·4)** complex, which in turn is related to the fluorescence of the complex. The amount of fluorescence contribution from the complex at a given time, which is associated with the fraction of the substrate depurinated, can be calculated from equations 1 and 2. The apparent rate constant (k_{ap}) was determined by fitting the fluorescence data using an exponential rise equation 3 (Fig. S3).[S4]

$$I_t = I_L + I_C \qquad (1)$$

$$I_L = I_{L0} - \{(I_{L0}-I_t)/(I_{L0}-I_S)\}I_{L0} \qquad (2)$$

I_t = fluorescence intensity at time = t

I_L = fluorescence intensity of ATMND ligand at time = t

I_C = Fluorescence intensity of ligand-product duplex (**2·4)** complex at time = t

I_{L0} = fluorescence intensity of ATMND ligand at t = 0 min

I_S = fluorescence intensity at saturation point

$$y = a + b(1-e^{-k_{ap}t}) \qquad (3)$$

y is fractional depurination, k_{ap} is apparent rate constant, t is time, a and b are coefficients, respectively.

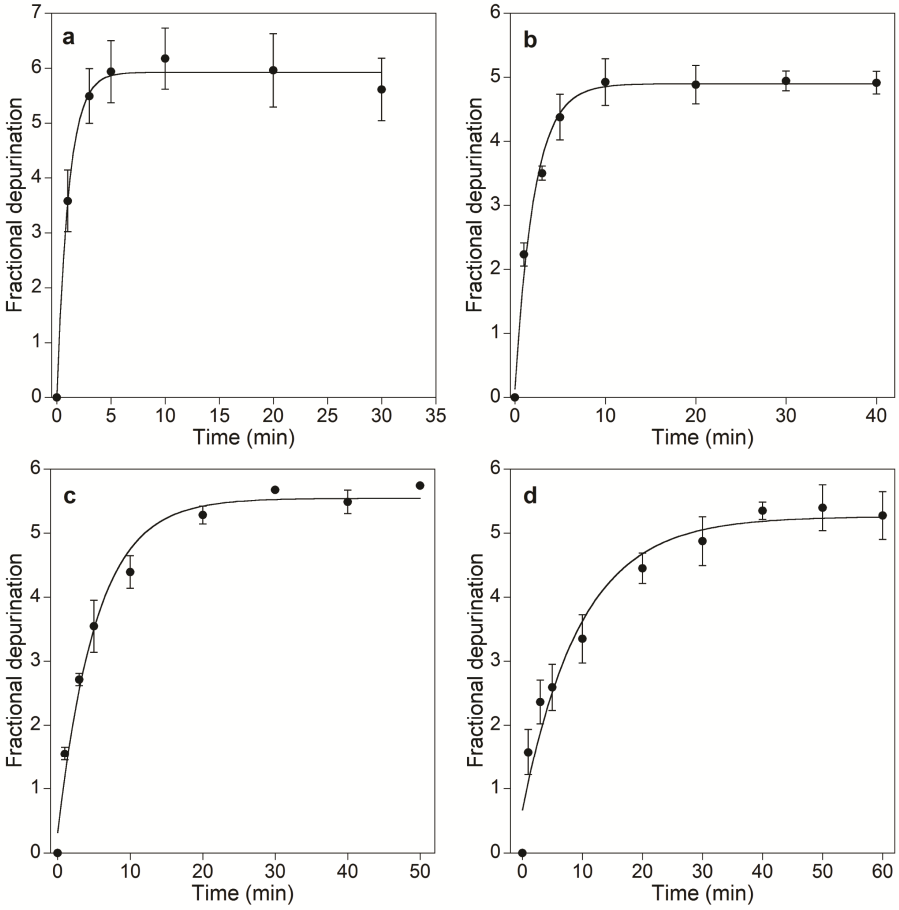

Fig. S3 Kinetic profiles of the depurination of oligoribonucleotide substrate **1** in the presence of saporin (a) 4 μM, (b) 2 μM, (c) 0.5 μM and (d) 0.25 μM. Rate constants were determined from fraction depurination vs. time plots. Curve fits are shown in solid lines. R values were very close to unity. See section 6 for details.

Table S1. Apparent rate constant (k_{ap}) of depurination of RNA **1** in the presence of varying concentration of saporin.[a]

[saporin] (μM)	k_{ap} (min^{-1})
4.00	0.92 ± 0.09
2.00	0.47 ± 0.05
0.50	0.19 ± 0.02
0.25	0.10 ± 0.02

[a] Concentration of RNA **1** (2 μM). See sections 5 and 6 for details.

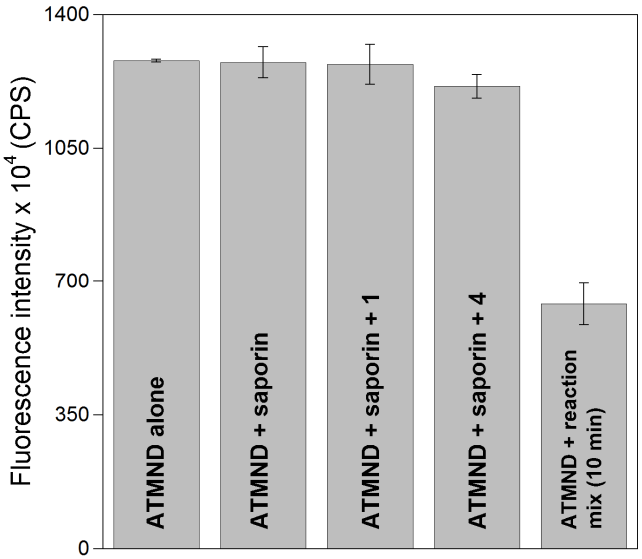

Fig. S4 Effect of saporin, saporin and **1**, and saporin and **4** on ATMND fluorescence in Tris-cacodylate buffer combination (pH 7.2). Final concentration of saporin, **1**, **4** and ATMND are 1 μM, 0.5 μM, 0.55 μM and 100 nM, respectively. Samples were excited at 350 nm with excitation and emission slit widths of 6 and 9 nm, respectively. Fluorescence of ATMND was measured at λ_{em}=406 nm. For experimental details see section 5.

7. References

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