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## Multivalent Inhibition of the *Aspergillus fumigatus* KDNase

Mathieu Scalabrini,<sup>a</sup> Denis Loquet,<sup>a</sup> Camille Rochard,<sup>b</sup> Mélyne Baudin-Marie,<sup>a</sup> Coralie Assailly,<sup>a</sup> Yoan Brissonnet,<sup>a</sup> Franck Daligault,<sup>c</sup> Amélie Saumonneau,<sup>c</sup> Annie Lambert,<sup>c</sup> Cyrille Grandjean,<sup>c</sup> David Deniaud,<sup>a</sup> Paul Lottin,<sup>d</sup> Sagrario Pascual,<sup>d</sup> Laurent Fontaine,<sup>d</sup> Viviane Balloy<sup>b</sup> and Sébastien G. Gouin<sup>a\*</sup>

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*Aspergillus fumigatus* is a saprophytic fungus and opportunistic pathogen often causing fatal infections in immunocompromised patients. Recently *Af*KDNase, an exoglycosidase hydrolyzing 2-keto-3-deoxynononic acid (KDN), a rare sugar from the sialic acid family, was identified and characterized. The principal function of *Af*KDNase is still unclear, but a study suggest a critical role in fungal cell wall morphology and virulence. Potent *Af*KDNase inhibitors are required to better probe the enzyme's biological role and as potential antivirulence factors. In this work, we developed a set of *Af*KDNase inhibitors based on enzymatically stable thio-KDN motifs. C2, C9-linked heterodi-KDN were designed to fit into unusually close KDN sugar binding pockets in the protein. A polymeric compound with an average of 54 KDN motifs was also designed by click chemistry. Inhibitory assays performed on recombinant *Af*KDNase showed a moderate and strong enzymatic inhibition for the two classes of compounds, respectively. The Poly-KDN showed more than a nine hundred fold improved inhibitory activity ( $IC_{50} = 1.52 \pm 0.37 \mu M$ , 17-fold in a KDN molar basis) compared to a monovalent KDN reference, and is to our knowledge, the best synthetic inhibitor described for a KDNase. Multivalency appears to be a relevant strategy for the design of potent KDNase inhibitors. Importantly, poly-KDN was shown to strongly decrease filamentation when co-cultured with *A. fumigatus* at micromolar concentrations, opening interesting perspectives in the development of antivirulence factors.

### Introduction

*Aspergillus fumigatus* is an environmental ubiquitous fungus thriving in the soil and feeding on organic decaying matter. It can acts as an opportunistic pathogen, often causing fatal infections in immunocompromised hosts.<sup>1</sup> *A. fumigatus* heavily sporulates small-sized conidia that are easily inhaled into the lower respiratory tract, where they germinate into hyphae and cause various lung diseases such as invasive aspergillosis or allergic bronchopulmonary aspergillosis.<sup>2</sup> Invasive aspergillosis is now a major cause of death for individuals with haematological malignancies, solid organ transplant recipients, and hematopoietic stem cells transplant patients, despite conventional treatments with triazole antifungals or amphotericin B.<sup>3</sup> New chemotherapeutics targeting virulent factors of the fungus are urgently needed to manage *A. fumigatus* infection.

Sialidases (SAs) or neuraminidases, are glycoside hydrolases hydrolysing sialic acids, which are negatively-charged sugars widely present in animal species. Bacterial, parasitic and viral SAs have been reported as virulent factors used by the pathogens to i) feed on host sialic acids, ii) unmask binding ligands for cell colonization, or iii) form protective biofilms.<sup>4</sup> SA inhibitors may be relevant anti-infective agents,<sup>5</sup> as illustrated by the approval of zanamivir (Relenza) and oseltamivir (Tamiflu), two anti-influenza drugs preventing virion release from infected cells. Academic research also focuses on developing selective inhibitors of human SAs,<sup>6,7</sup> to impact cellular processes, and bacterial and parasitic SAs,<sup>8,9</sup> as an alternative to conventional antimicrobial treatments facing increasing resistances. Moore and co-workers discovered a gene encoding for a putative sialidase (*Af*SA) in the genome sequence of *A. fumigatus*.<sup>10</sup> In comparison to bacterial SAs, *Af*SA showed poor efficiency in cleaving the *N*-acetylneuraminic acid (Neu5Ac) substrates and was also poorly inhibited by the SA transition state inhibitor 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (Neu5Ac2en).<sup>11</sup> 2-keto-3-deoxynononic acid (KDN), a member of the sialic acid family with an hydroxyl group in C5 instead of an acetamide, was shown to be the preferred enzymatic substrate. Subsequent studies confirmed that *Af*SA is a KDNase (*Af*KDNase) with a suggested nutritional role in the life cycle of the fungus. While SAs are widely studied virulent factors much less is known about KDNases. Although KDN is frequently found in bacteria

<sup>a</sup> Nantes Université, CNRS, CEISAM, UMR 6230, F-44000 Nantes, France.

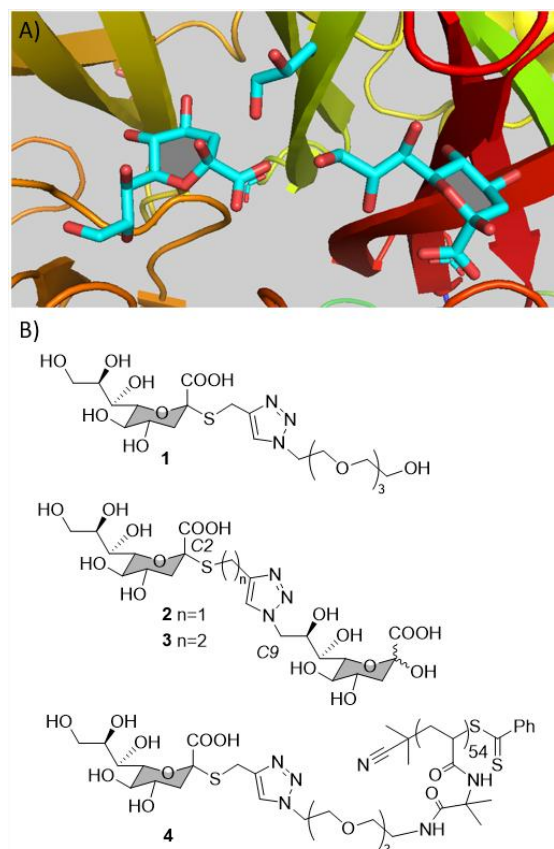
<sup>b</sup> Sorbonne Université, Inserm, Centre de Recherche Saint-Antoine, CRSA, Paris, France,

<sup>c</sup> Nantes Université, CNRS, US2B, UMR 6286, F-44000 Nantes, France 2 rue de la Houssinière, BP 92208, 44322 Nantes Cedex 3, France,

<sup>d</sup> Institut des Molécules et Matériaux du Mans (IMMM), UMR 6283 CNRS, Le Mans Université, Av. O. Messiaen, 72085 Le Mans cedex 9, France.

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and lower vertebrates, only trace amounts were found in specific cancerous and normal human tissues.<sup>12,13</sup> Here, its proportion relative to total sialic acid is below 1%, questioning the role of the AfKDNase in the fungus's pathogenicity. Moore and co-workers developed a AfKDNase knockout strain of *A. fumigatus* that showed reduced growth on KDN medium, abnormal cell wall morphology, and attenuated virulence with a lower fungal burden in animal treated by amphotericin B.<sup>14</sup> These results highlight the importance of the enzyme for the fungus's cell wall integrity and virulence, suggesting that inhibitors might have therapeutic potential for invasive aspergillosis treatments in combination with amphotericin B. In this study, we developed a series of synthetic inhibitors of AfKDNase based on KDN analogues. The previously reported AfKDNase crystal structure complexed with KDN revealed distinctive and intriguing interaction patterns.<sup>11</sup> The active site of the protein featured a bound KDN residue and a second KDN sugar in close proximity (Figure 1A). This unique feature may be associated with the enzymatic preference for natural poly-KDN substrates. Notably, the short distance of 6.4Å from the anomeric oxygen to the C9 of the second KDN led use to design potential inhibitors **2** and **3**, where two KDN residues are linked through a short spacer between the C2 and C9 atoms (Figure 1B). This approach, part of a fragment-optimization strategy, aimed to enhance binding enthalpies. It is worth mentioning that two additional KDN residues were found to be bound at a remote distance from the catalytic site.<sup>11</sup> Building on this, we hypothesized that a polymer of KDN sugars, as exemplified by compound **4**, might exhibit enhanced inhibitory effects. This could occur through simultaneous interactions in the KDN binding sites (chelate effect) or via internal diffusion of the polymeric chain in a zip-like fashion within the binding sites.<sup>15</sup> We previously reported similar synergistic (or multivalency) effects in related bacterial SAs, where substrate or transition state analogues clustered on polymeric scaffolds demonstrated inhibitory activities improved by up to four orders of magnitude.<sup>16,17</sup> For this investigation, compound **1** will serve as a monovalent reference to evaluate the advantages of KDN heterodimerization (**2** and **3**) and polymerization (**4**). To prevent hydrolysis of the sugar motifs in **1-4** by AfKDNase, we substituted the anomeric oxygen with sulfur atom. This modification aims to limit the hydrolytic efficiency of AfKDNase, as demonstrated by the lower hydrolysis rate of the S analogue of the 4-methylumbelliferyl KDN-glycoside substrate.<sup>18</sup>

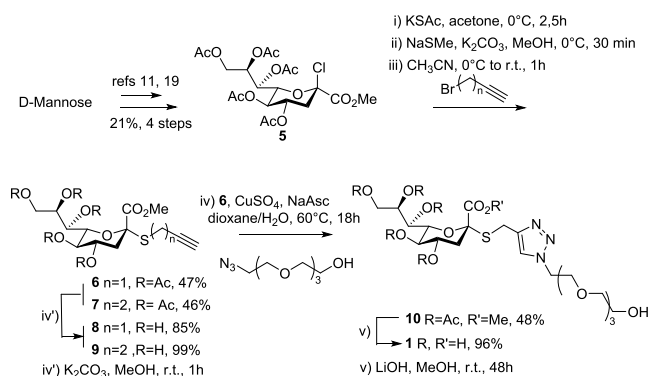


**Figure 1.** A) Crystal structure of AfKDNase (PDB code = 2XZI) displaying two KDN in close proximity with a glycerol molecule (cryoprotectant). B) Structure of the synthesized compounds **1-4**.

## Results and Discussion

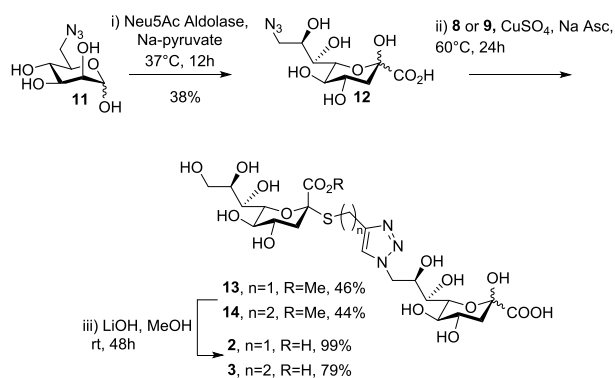
The chemical synthesis of **1-4** started with the previously described compound **5** (Scheme 1).<sup>11,19</sup> Briefly, mannose was initially converted to KDN sugar by Neu5Ac aldolase enzyme in presence of sodium pyruvate. Following the methylation of the carboxylic acid in methanol with acidic resin, the hydroxyl groups were protected in a mixture of acetic anhydride and pyridine. The anomeric acetate of the corresponding compound was then substituted by a chlorine atom in an HCl-saturated acetyl chloride solution to yield **5**, achieving an overall yield of 23% for the four steps. The introduction of a sulfur atom was accomplished through nucleophilic substitution with potassium thioacetate. Despite initial challenges in selectively deprotecting the thioacetate group with sodium methanolate at low temperatures,<sup>20</sup> optimization efforts led to the best results using a mixture of sodium methanethiolate and potassium carbonate, followed by rapid alkylation with propargyl or butynyl bromide, yielding **6** and **7**, respectively. The hydroxyl deprotections of these two

compounds with  $K_2CO_3$  resulted in the formation of **8** and **9**, which were retained for the synthesis of the heterodimers. Copper-catalyzed azide-alkyne cyclization (CuAAC) with **6** and azido-tetraethyleneglycol (N<sub>3</sub>-TEG) produced the single 1,4-disubstituted triazole **10**. This compound was identified through the significant  $\Delta(\delta C-4 - \delta C-5)$  value observed in the <sup>13</sup>C NMR spectra for the triazole carbon atoms.<sup>21</sup>



**Scheme 1.** A) Chemical synthesis of monovalent reference **1**.

The di-KDN derivatives **2** and **3** were designed as presented in scheme 2. Compound **11** was first synthesized from mannose as described in literature.<sup>22</sup> Leveraging the substrate promiscuity of the Neu5Ac aldolase enzyme, an efficient conversion of **11** into 9-azido-9-deoxy-KDN **12** was achieved in the presence of sodium pyruvate.<sup>23</sup> This unprotected compound was then subjected to a CuAAC reaction with **8** and **9**, resulting in the formation of **13** and **14**, which were subsequently purified by chromatography. The final step involved deprotecting the methyl esters with LiOH, yielding pseudo-disaccharides **2** and **3**. The synthesis of polymer **4** was accomplished through a CuAAC reaction between **6** and a previously described azido-functionalized polymer **15** (Figure S1).<sup>17</sup>



**Scheme 2.** Chemical synthesis of pseudo-disaccharides **2** and **3**.

The inhibitory capacity of **1-4** was subsequently assessed against AfKDNase. Recombinant enzyme production and purification (Figure S2)<sup>10,24</sup> as well as the chemical synthesis of the pro-fluorescent substrate (4-methylumbelliferyl)-3-deoxy-D-glycero-D-galacto-non-2-ulopyranosonic acid (MU-KDN),

were performed following previously described procedures.<sup>11</sup> AfKDNase activity was monitored by measuring the fluorescence emission of 4-methylumbelliferone (MU,  $\lambda_{ex}$  = 365 nm and  $\lambda_{em}$  = 450 nm) after MU-KDN hydrolysis. The AfKDNase affinity for the MU-KDN substrate ( $K_m$  = 575 ± 53 μM) was consistent with the previously reported value ( $K_m$  = 230 μM).<sup>11</sup> The inhibitory activities of **1-4** ( $IC_{50}$ ) were determined through Dixon plot analysis (Figure S3) and are presented in Table 1. The relative inhibitory potency of **2-4** compared to the monovalent reference **1** was calculated as follows ( $RP = IC_{50}^1 / IC_{50}^{2-4}$ ). Additionally, we divided the RP by the number of KDN units (n) in the molecules (RP/n). A significant synergistic (or multivalent) inhibitory effect is observed if  $RP/n > 1$ , reflecting the improvement of inhibitory capacity per KDN unit of the molecule. Monovalent **1** demonstrated poor AfKDNase inhibition with an  $IC_{50}$  of 1420 ± 90 μM. However, heterodimeric compounds **2** and **3** exhibited improvement in inhibition compared to **1**, showing moderate  $IC_{50}$  values of 453 ± 130 and 526 ± 100 μM, respectively. Importantly, polymeric compound **4** emerged as a potent inhibitor of the AfKDNase with a low  $IC_{50}$  of 1.52 ± 0.37 μM. Compound **4** demonstrated a 934-fold improvement in inhibitory capacity compared to **1**, corresponding to a significant enhancement factor for each clustered KDN of  $RP/n = 17.3$  fold.

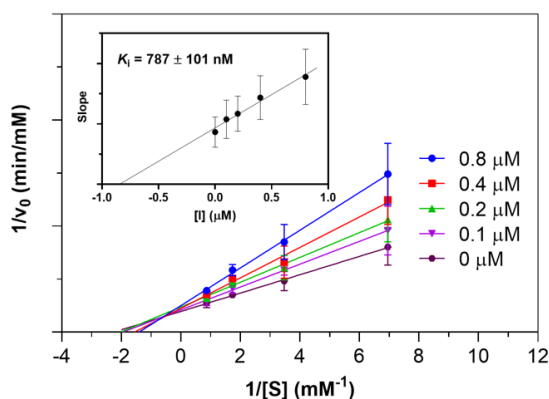
**Table 1.** Inhibitory concentrations ( $IC_{50}$ ), relative inhibitory potencies (RP), and valency-corrected relative inhibitory potencies (RP/n) of compounds **1-4** against AfKDNase. Average values from 3 independent experiments.

Cpds	Val	$IC_{50}$ (μM)	RP	RP/n
1	1	1420 ± 90	1	1
2	2	453 ± 130	3.1	1.6
3	2	526 ± 100	2.7	1.4
4	54	1.52 ± 0.37	934	17.3

Note: The valency-corrected relative inhibitory potency (RP/n) is calculated by dividing RP by the number of KDN units in the molecules. A significant synergistic (or multivalent) inhibitory effect is indicated if  $RP/n > 1$ , reflecting improved inhibitory capacity per KDN unit in the molecule (Val).

We also determined the inhibitory constant ( $K_i$ ) and valency-corrected inhibitory constant ( $K_i^{VC}$ ) of polymer **4**. Lineweaver-Burk plots analysis (Figure 2) performed in triplicate indicate a low  $K_i$  of 784 ± 101 nM ( $K_i^{VC} = 42$  μM), consistent with the low  $IC_{50}$ . The conventional SA transition state inhibitor Neu5Ac2en was shown to poorly inhibit KDNases,<sup>11</sup> and to our knowledge, the most potent competitive inhibitor to date is the KDN2en analogue, with a hydroxyl group at C5 instead of an acetamide. Reported  $K_i$  values for this compound against *A. fumigatus*, *A. terreus* and *T. rubrum* KDNases are between 100 and 160 μM,<sup>25</sup> which means that **4** is significantly a more potent inhibitor than KDN2en. While multivalency effects have been extensively reported for carbohydrate-binding proteins (lectins),<sup>26,27</sup> they remain poorly exemplified on biologically relevant glycoside hydrolases.<sup>28-30</sup> Furthermore, dendritic and polymeric Neu5Ac derivatives were previously designed for blocking viral and bacterial hemagglutinins or SAs,<sup>16,17,31-34</sup> but, to our knowledge, no examples of synthetic multivalent KDN inhibitors against KDNases has been reported to date. The

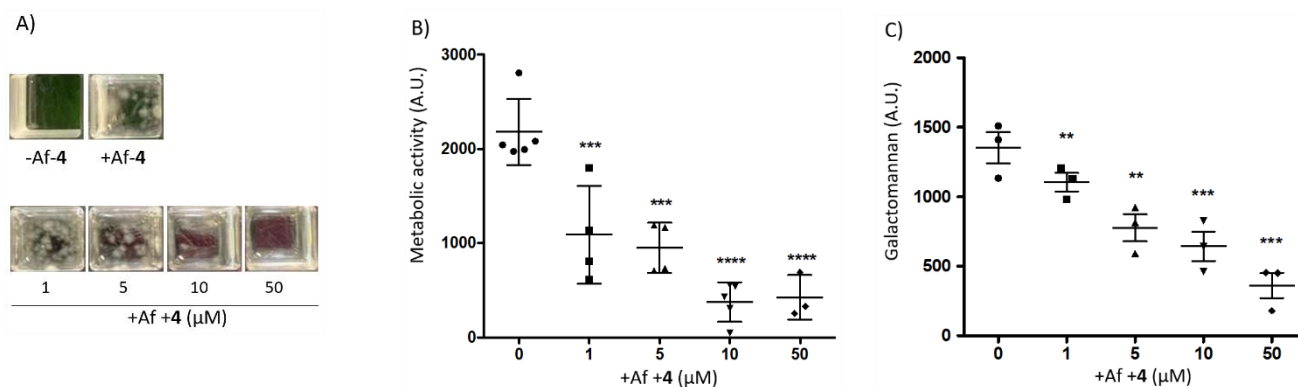
results presented here suggest that the multivalent concept is a relevant strategy to design potent KDNase inhibitors.



**Figure 2** Lineweaver-Burk plots for the inhibition of *AfKDNase* by compound **4** and the secondary plots for  $K_i$ . Data were obtained from 15 min incubation with MU-KDN in absence or presence of **4** (0-0.8  $\mu\text{M}$ ). All data are mean of triplicates.

As previously mentioned, *AfKDNase* knockout strain of *A. fumigatus* displayed abnormal morphology and reduced

growth.<sup>14</sup> We were curious about the potential impact of *AfKDNase* inhibition on conidia germination and filamentation of clinical strains of *A. fumigatus*. Polymer **4**, the most potent inhibitor of the series, was co-cultured with *A. fumigatus* conidia at concentrations ranging from 1 to 50  $\mu\text{M}$  (expressed in mol of KDN). Macroscopic examinations revealed a significant decrease in filamentation levels when the concentration of **4** exceeded 10  $\mu\text{M}$  (Figure 3A). The antifungal activity was further quantified using two independent methods i) measuring the metabolic activity of *A. fumigatus* (Figure 3B) and ii) assessing the level of galactomannan in the supernatant, a polysaccharide released during *A. fumigatus* growth (Figure 3C). Resazurin conversion to fluorescent resorufin<sup>35</sup> was used as a reporter of metabolic activity of *A. fumigatus* treated or not by a range of concentrations of **4**. Galactomannan antigen quantification was determined by using a Platelia Aspergillus EIA (Bio-Rad) kit.<sup>36</sup> Results clearly demonstrated a substantial decrease in both metabolic activity and fungal galactomannan release in the presence of **4** in a dose-dependent fashion. At a concentration of 50  $\mu\text{M}$  of polymer, metabolic activity and galactomannan levels were only 20% and 27% of the initial values, respectively. Interestingly, this fungistatic effect was observed in three different culture media (Sabouraud and F-12 Nutrient Mixture from Thermo Fisher complemented or not with fetal calf serum). These preliminary findings suggest that polymeric inhibitors of *AfKDNase*, such as **4**, may strongly impact *A. fumigatus* development. Additional assays are underway to understand the detailed mechanism of inhibition and the potential of *AfKDNase* antagonists to attenuate fungal virulence.



**Figure 3** A) Macroscopic observations showing a decreased filamentation with **4**.  $10^2$  to  $10^3$  *A. fumigatus* conidia were incubated in Sabouraud at 37°C with different concentrations of **4**. After 15 hours, fungal growth was analyzed macroscopically B) metabolic activity of *A. fumigatus*.  $10^4$  *A. fumigatus* conidia were incubated in Sabouraud with different concentrations of **4** in presence of 0.001 % Resazurin and incubated for 15 hours, at 37°C. Fluorescence was measured and metabolic activity expressed in arbitrary units (A.U.). C) level of galactomannan in the supernatant.  $10^4$  *A. fumigatus* conidia were incubated in Sabouraud for 15 hours at 37°C with different concentrations of **4**. Galactomannan release was measured and expressed in arbitrary units (A.U.). Statistical analysis were performed using analysis of variance, followed by Bonferroni multiple comparison test. \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$

## Conclusions

KDNases are intriguing enzymes belonging to the glycoside hydrolase family 33 (GH 33). While the presence of KDN sugar in humans is minimal, experiments with *AfKDNase* knockout strains underscore the enzyme's crucial role in

normal fungal growth and virulence.<sup>14</sup> So far, synthetic inhibitors of KDNases remain unexplored, representing a promising avenue for gaining insights into their enzymatic functions and potentially developing complementary treatments for fungal infections.

In this study, we successfully developed a series of synthetic KDNase inhibitors based on KDN substrates. Heterodimeric

compounds **2** and **3** exhibited moderate levels of enzyme inhibition, serving as interesting precursors for the development of more potent ligands through fragment-based approaches. Notably, compound **4** emerged as the most potent non-covalent inhibitor of a KDNase reported to date. Intriguingly, **4** also demonstrated a significant inhibitory effect on *A. fumigatus* growth. Although the precise role of AfKDNase in fungal pathogenicity is yet to be fully elucidated, the robust antifungal effect observed with compound **4** opens up possibilities for the development of optimized KDN glycoclusters as potential anti-virulence factors against *A. fumigatus* infections. This work paves the way for further exploration of KDNase inhibitors and their therapeutic potential in combating fungal diseases.

## Author Contributions

S.G. conceived the initial idea; S.G., D.D. and F.D. supervised the chemical synthesis and inhibition assays; M.S., M B-M, D.L., C. A., Y.B. performed the chemical synthesis and inhibition assays; Under the guidance of S.P. and P.L., L.F. performed the synthesis of the azido-functionalized polymer; Under the guidance of F.D. and C.G., A.S. and A.L. produced AfKDNase; Under the guidance of V.B., C.R. performed the biological assays and analysis; S.G. wrote the manuscript.

## Conflicts of interest

There are no conflicts to declare.

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