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Human α-Defensins Neutralize Toxins of the Mono-ADP-Ribosyltransferase Family

Running title: Neutralization of Bacterial Toxins as Prominent Defense Mechanism of α-Defensins

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Various bacterial pathogens secrete toxins, which are not only responsible for fatal pathogenesis of disease, but also facilitate evasion from host defenses. One of the best known bacterial toxin groups is the mono-ADP-ribosyltransferase family. Here, we demonstrate that human neutrophil α-defensins are potent inhibitors of the bacterial enzymes, particularly against diphtheria toxin (DT) and *Pseudomonas* exotoxin A (ETA). Human neutrophil protein 1 (HNP1) inhibited DT- or ETA-mediated ADP-ribosylation of eukaryotic elongation factor 2 (eEF2) and protected HELA cells against DT- or ETA-induced cell death. Kinetic analysis revealed that inhibition of DT and ETA by HNP1 was competitive with respect to eEF2 and uncompetitive against NAD^+ substrates. Our data reveal that toxin neutralization represents a novel biological function of HNPs in host defense.

Key words: Human neutrophil protein 1, Diphtheria toxin, *Pseudomonas* exotoxin A

INTRODUCTION

Mono-ADP-ribosylation, a covalent chemical modification process, is not only a component of host cell intracellular signaling but is also a widely used mechanism of bacterial pathogenesis [1]. This reaction is catalyzed by mono-ADP-ribosyltransferases (ART), which transfer the ADP-ribose moiety of NAD^+ to a target protein with nicotinamide release [2]. This enzyme family is structurally classified into two groups: the cholera toxin (CT) group and the diphtheria toxin (DT) group [3, 4]. Although these two groups do not share any significant sequence homology, they show conserved secondary and tertiary structural elements. The CT group bears an NAD^+ binding pocket characterized by the catalytic Glu, a β/α structural element, and an Arg residue. The majority of bacterial ARTs and mammalian mono-ARTs belong to this group. On the other hand, the DT group including DT and ETA comprises a conserved Glu residue, the β/α motif and a His residue instead of Arg. It is well established that DT and ETA cause cell death by inactivating eEF2 via ADP-ribosylation [5].

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A recent study showed that mammalian ART-1 modifies Arg-14 of human neutrophil protein 1 (HNP1) [6]. HNPs are small cationic peptides, which belong to the α-defensin family. They are structurally characterized by their β-sheet dominant structure and intramolecular disulfide bridges [7]. HNP1-3 are identical in sequence in all but their N terminal residues [8]. *In vitro*, these peptides display microbicidal activity against a broad spectrum of bacteria, fungi, and viruses [7]. Recently, we showed that HNP1-3 inhibit the activity of lethal factor (LF), a metalloprotease produced by *Bacillus anthracis* [9]. Herein, we report another example of neutralization of bacterial toxins by HNPs: HNP1-3 neutralize bacterial ARTs, particularly, DT and ETA of the DT group but not members of the CT group. Together with our previous findings that HNP1-3 neutralize LF of *B. anthracis*, our data reveal that toxin neutralization represents a novel biological function of HNP1-3 in host defense.

EXPERIMENTAL

Bacterial toxins and peptides

Murine ART-1 was purified from 293T cells transfected with pCDM8-mART1.Fc (a kind gift of Dr. Friedrich Nolte, University of Hamburg) as described [10]. All bacterial toxins were purchased from (Quadratech, UK). For kinetic experiments, the catalytic fragment of ETA with a C-terminal 6-His tag (ETAc), the catalytic fragment of DT with a C-terminal 6-His tag (DTA), and *S. cereviseae* eEF2 were purified as described previously [11-13]. Synthetic HNP1 was obtained from Bachem (Weil am Rhein, Germany). Natural HNP1-3 was purified from human buffy coats (German Red Cross) as described [14]. Synthetic LL-37 was generously provided by Dr. Hubert Kalbacher (University of Tübingen).

ART assay

ADP-ribosylation of HNP1 was analyzed as described [6]. In brief, 10 μg HNP1 was incubated in 200 μl of 50 mM potassium phosphate buffer (pH 7.5), with 100 ng of each ART (activated) and 3 mM NAD^+ at 30 °C. After overnight incubation, the reactions were applied to a XTerra® C18 reverse-

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phase HPLC column (Waters, Milford MA) and analyzed. Activation of each toxin was performed as previously described [15-19]. In order to remove DTT, which will cause linearization of HNP1, the buffers for toxin activation were modified by using a NAP-10 desalting column (Amersham, UK) before each experiment. Pertussis toxin A promoter was obtained from List biological laboratories (Quadratech, UK).

ADP-ribosylation of eEF2 was examined as follows. Three μg of HELA cell lysates, NAD^+-P^{32}, and 1 ng of activated DT or ETA were incubated in 20 mM Tris-HCl (pH.8.0) and 1 mM EDTA in the presence of HNP1 or LL-37 at room temperature for 30 min. The reactions were terminated by adding SDS-loading buffer and analyzed by SDS-PAGE.

Toxin protection assay

One day before the assay, HELA cells were seeded in 96-well plates at a density of 1.5×10^4 cells per well in 100 μl RPMI 1640 medium containing 10% fetal calf serum (FCS). For this assay, 20 ng/ml DT or 100 ng/ml ETA, and described amounts of HNP-1 or LL-37 were added simultaneously to cells in serum-free RPMI 1640. Twenty-four hours after treatment, cell viability was determined by methyl thiazole tetrazolium (MTT) assay.

Binding assay and kinetic analysis

The quenching of the intrinsic tryptophan fluorescence of toxins was used to determine the binding constant (K_d) for HNP1 with both DTA and ETAc as described elsewhere [20].

In order to determine IC_{50} values, substrates, ε-NAD^+ and eEF2, at saturating doses of 500 μM and 14 μM, respectively, and various concentrations of HNP1 (0–20 μM) were added to the assay medium (20 mM Tris-HCl, pH 7.9) in a 70 μl total volume. The reaction mixture was incubated for 5 min at 25°C and initiated by adding 10 nM (final concentration) ETAc or DTA. The reaction was monitored as previously described [21].

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The NAD^+-dependent and eEF2-dependent ART activities of the toxins were measured as reported previously [21, 22]. In brief, the NAD^+-dependent ART assay was performed with the eEF2 concentration in the assay medium held to 14 μM, while the concentration of ε-NAD^+ varied between 0 and 500 μM in the presence of HNP1 at 0, 5, 10, 15, and 20 μM. The reactions were initiated with 20 nM (final concentration) ETAc or DTA. The eEF2-dependent ART assay was performed at 500 μM ε-NAD^+ while varying eEF2 substrate (0–15 μM) in the presence of HNP1 at 0, 5, 7.5, and 10 μM. The data were analyzed by linear regression analysis of both the Hanes-Woolf and the Lineweaver-Burk (LB) plots. The K_i values were determined using Dixon plots, as well as from secondary plots of the slope of the LB plots versus inhibitor concentration.

Infection assay

One day before the assay, HELA cells were seeded in 96-well plates at a density of 2×10^4 cells per well in 100 μl RPMI 1640 medium containing 10% fetal calf serum (FCS). For the assay, 4×10^5 *Corynebacterium diphtheriae* cells (ATCC #13812) were added per well and the described amounts of HNP-1 were then added to the cells in serum-free RPMI 1640. Six hours after infection, bacteria were killed by Penicillin and Streptomycin and cells were further incubated for 24h. Cytotoxicity was determined by the CytoTox 96® cytotoxicity assay (Promega).

RESULTS and DISCUSSION

HNP1 is not a substrate of bacterial ARTs

It has been shown recently that HNP1 is a substrate of mammalian ART-1 [6]. Therefore, we first examined whether bacterial ARTs were also able to transfer ADP-ribose to HNP1. CT and pertussis toxin (PT) were tested as representatives of the CT group, and DT and ETA as those of the DT group. Consistent with previous findings [6], we found distinct absorbance peaks in HNP1 incubated with mouse ART-1 corresponding to the ADP-ribose HNP-1 product(s) (Figure 1, denoted by arrows). In

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contrast, HNP1 treated with bacterial ARTs showed the same profile as the control (HNP1 without treatment), i.e., no ADP-ribose product was observed (Figure 1). We conclude that HNP1 does not serve as a substrate for these bacterial ARTs.

HNPs protect cells from DT- or ETA-mediated cell death

Next, we determined whether HNPs had any effect on bacterial ART- treated cells. HELA cells treated with DT or ETA succumbed to these toxins within 24 h. In marked contrast, the addition of HNP1 completely abolished cytotoxicity in a dose-dependent manner (Figures 2A and 2B). Purified natural HNP1-3 mixtures from human leukocytes showed similar protective capabilities (data not shown) whereas LL-37, another neutrophil cationic peptide with similar size and net charge as HNP1-3, did not display any significant effect (Figures 2A, and 2B).

We also examined the effect of HNPs on CT- or PT- treated Chinese hamster ovary (CHO) cells. However, we did not detect any significant difference of cAMP levels between toxin only treated groups and toxin and HNPs treated groups, indicating HNPs do not prevent CT- or PT-mediated intoxication in CHO cells (data not shown).

HNP1 inhibits ADP-ribosylation of eEF2

To determine whether HNP1 directly inhibits ART activity of DT and ETA, we examined the transfer of radiolabelled ADP-ribose from NAD^+ to eEF2 *in vitro*. The eEF2 in HELA cell extracts was efficiently ADP ribosylated after incubation with activated DT or ETA. In contrast, ADP-ribosylation of eEF2 was inhibited in the presence of HNP1 in a dose-dependent manner. LL-37 had no significant effect on this reaction suggesting that the inhibition by HNP1 was specific (Figure 3). Surprisingly, linearized HNP1 also showed comparable inhibition activity with intact HNP1. HNP1 contains three intramolecular disulfide linkages to maintain its stable and compact conformation. Our previous study suggested that the unique structure of HNP1 determined by disulfide bonds is a crucial requirement

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for inhibition against anthrax LF activity [9]. In contrast to LF, DT and ETA were efficiently inhibited by DTT-treated HNP-1 indicating that DT and ETA recognize the primary sequence of HNP-1 rather than a structural motif in HNP-1.

HNP1 inhibits competitively against eEF2 and uncompetitively against NAD^+

Figures 4A and 4B show the fluorescence quench curves for the intrinsic tryptophan fluorescence of ETAc (catalytic fragment of ETA with C-terminal His_6 tag) and DTA (catalytic fragment of DT with C-terminal His_6 tag) on titration with HNP1 as the ligand. In this assay, HNP1 appears to bind to DTA more tightly (K_d = 6.8 ± 1.2 μM) than to ETAc (K_d = 21.5 ± 2.1 μM).

To elucidate the mode of action, we characterized the kinetics of inhibition of HNP1 against both DTA and ETAc. The initial rates of enzyme reaction in the presence of various concentrations of HNP1 provided an IC_{50} value of 11.1 ± 2.7 μM against DTA and 19.2 ± 6.5 μM against ETAc (Figures 5A and 5B). In competition assays, when the concentration of eEF2 was held constant and that of NAD^+ was varied, HNP1 was identified as an uncompetitive inhibitor against both toxins, invoking a change in both K_M and V_{max} (Figures 6A and 6B). On the other hand, when the concentration of NAD^+ was fixed and that of eEF2 was varied, HNP1 acted as a competitive inhibitor (Figure 6C and inhibition data against DTA not shown). Kinetic and thermodynamic parameters for the interaction of HNP1 with both DTA and ETAc in this study are summarized in Table 1. These data show that HNP1 is a more effective inhibitor against DTA than ETAc (IC_{50} values: 11.1 ± 2.6 and 19.2 ± 6.5 μM, respectively; Ki values with variable NAD^+: 7.3 ± 1.7 and 13.3 ± 0.9 μM, respectively), which correlates well with the data from the HELA cell protection assay (Figure 2).

Our kinetic studies indicate that HNP1 is a competitive inhibitor against both DT and ETA with respect to eEF2. Mammalian ART-1 has been shown to transfer ADP-ribose to HNP1 [6]. As a corollary, HNP1 would be expected to recognize and bind to the eEF2 docking site within both DT and ETA. It is, therefore, not surprising that HNP1 is a competitive inhibitor against the eEF2

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substrate for the toxins. On the other hand, HNP1 acted as an uncompetitive inhibitor for NAD^+ substrates for the toxins. An uncompetitive inhibitor is defined as an inhibitor binding to the enzyme–substrate (ES) complex but not to the free enzyme (E). Although HNP1 bound to DT and ETA in the absence of NAD^+ (Figures 4A and B), it was identified as an uncompetitive inhibitor with respect to NAD^+ in our assays. The following explanation is a plausible rationalization of this observation. It is possible that HNP1 binds to the free E in a unique manner without affecting the binding of NAD^+. Yet, once NAD^+ is accommodated in the E-binding pocket, HNP1 may bind to the ES complex in an inhibitory mode, which results in a kinetically impaired E (lower V_{max} and higher K_M). It remains to be elucidated whether unique HNP1-3 binding pockets exist in such functionally distinct bacterial toxins as LF [9], DT, and ETA.

HNP1 Rescues HELA cells from *C. diphtheriae*-induced cell death.

Having identified DT neutralization as a biological function of HNP1, we decided to examine whether HNP1 neutralizes DT that has been secreted by bacteria. HELA cells were infected with *C. diphtheriae* and were treated immediately with the indicated amounts of HNP1. As shown in Figure 7, we confirmed that HNP1 protects cells from *C. diphtheriae*-induced cell death in a dose-dependent manner.

Recently, we identified a new biological function of HNPs, namely neutralization of a secreted bacterial enzyme, the LF of *B. anthracis*. [9]. Consistent with, and in extension of our previous findings, here we show that HNP1-3 act as inhibitors of two additional bacterial toxins, DT and ETA. It is well known that these toxins are critical for the pathogenesis caused by *Corynebacterium diphtheriae* and *P. aeruginosa* [23]. These two toxins use molecular mimicry of an essential part of the eukaryotic host cell's rRNA in order to minimize the risk that the host could develop resistance against them. This is because mutations that might confer resistance to the host cell would also likely

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interfere with translation efficiency and fidelity in the host [24]. As an alternate defense strategy, the host has developed the capacity to neutralize these toxins by binding their enzyme domains with the α-defensins, HNP1-3. The HNP1-3 can be internalized into host cells [25] and the K_d of HNP1 for DT and ETA indicate that it can compete efficiently with eEF2 for association with these toxins inside host cells.

HNP1-3 have several advantages over known NAD^+ analogue inhibitors. First, HNP1-3 can prevent toxin binding to ribosomes by blocking the toxin's association with eEF2. It has been suggested that ART toxins can complex with eEF2 on the ribosomes [24, 26]. Since NAD^+ analogues fail to prevent toxins from binding to eEF2, the toxin may still be able to interfere with eEF2 on translational complexes. Second, HNP1-3 are more specific for the toxins because NAD^+ analogues will potentially inhibit various host metabolic enzymes such as dehydrogenases eventually becoming toxic to host cells.

Although HNPs are well known as antimicrobial peptides [27], emerging evidence suggests additional biological functions [28]. Many bacterial pathogens secrete enzymes into the host environment to avoid destruction by the immune system. Because recruited human neutrophils release abundant HNP1-3 locally in the inflammatory regions, we assume that HNP1-3 achieve efficient neutralization of bacterial enzymes *in vivo*. Hence we propose that neutralization of a group of bacterial toxins constitutes a major function of HNP1-3 in antimicrobial host defense.

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FIGURE LEGENDS AND TABLE

Figure 1 Separation of reaction products of HNP1 after incubation with mono-ARTs.

HNP1 was incubated with reaction buffer alone, murine ART-1 (mART-1), DT (diphtheria toxin), ETA (*Pseudomonas* exotoxin A), CT (cholera toxin) or PT (pertussis toxin) and then analyzed by RP-HPLC. The arrows indicate the peaks corresponding to the ADP-ribose-HNP1.

Figure 2 Effect of HNP1 on DT- or ETA- treated cells.

HELA cells were treated with DT (A) or ETA (B) in the presence of the indicated amounts of HNP1 or LL-37. Bacterial toxins and peptides were treated at the same time and incubated for 24 h. Cell viability was determined by the methyl thiazole tetrazolium (MTT) assay.

Figure 3 Effect of HNP1 on DT or ETA-mediated ADP-ribosylation of eEF2.

HELA cell lysates were incubated with DT (A) or ETA (B) in the presence of the indicated amounts of HNP1 or LL-37. The samples were analyzed by SDS-PAGE followed by autoradiography. In the presence of HNP1 at 0.1, 0.5, and 2.5 μM, DT activity decreased to 25, 8, and 0.5% of control DT activity and ETA activity declined to 37, 12, and 1% of control ETA activity, respectively.

Figure 4 Binding properties of HNP1 to DT and ETA.

The binding isotherm for HNP1 with DTA (A) or ETAc (B) was determined from the quenching of the intrinsic protein fluorescence. The raw fluorescence quenching data were converted to fractional saturation values [20] and plotted against the HNP1 concentration.

Figure 5 Inhibitory properties of HNP1 against DTA and ETAc.

The substrates, ε-NAD^+ and eEF2, in the fluorescence-based ART assay (see Materials and Methods)

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were used for examining the inhibitory properties of HNP1 against DTA (A) and ETAc (B). The concentration of the inhibitor that reduces the activity of the enzyme by 50%, the IC_{50} value, was determined by nonlinear regression curve fitting using Origin 6.1 (OriginLab, Northhampton, MA). The data were fit to the exponential first-order decay function.

Figure 6 Mode of inhibition of HNP1 against DTA and ETAc.

The NAD^+-dependent DTA (A) and ETAc (B) assays were performed with the fixed amount of eEF2 and variable concentrations of HNP1 and ε-NAD^+. The eEF2-dependent ETAc assay (C) was performed at a fixed concentration of ε-NAD^+ while varying amounts of eEF2 in the presence of HNP1. The data were analyzed by linear regression analysis of the Hanes-Woolf plot.

Figure 7 Protection of infected cells by HNP1

HELA cells were infected with *C. diphtheriae* and then treated with the indicated amounts of HNP-1. Cytotoxicity was determined by measuring lactate dehydrogenase levels released from HELA cells.

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TABLE 1 Kinetic and Thermodynamic Parameters for the Binding and Inhibition of Bacterial ART Activity by HNP1

Toxin	IC_{50} (μM)	Ki (μM)	K_d (μM)
	Variable NAD substrate: uncompetitive inhibition		
DT	11.1 ± 2.6	7.3 ± 1.7	6.8 ± 1.2
ETA	19.2 ± 6.5	13.3 ± 0.9	21.5 ± 2.1
	Variable eEF2 substrate: competitive inhibition		
DT		6.7 ± 1.1	
ETA		5.3 ± 0.4	

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Fig. 1

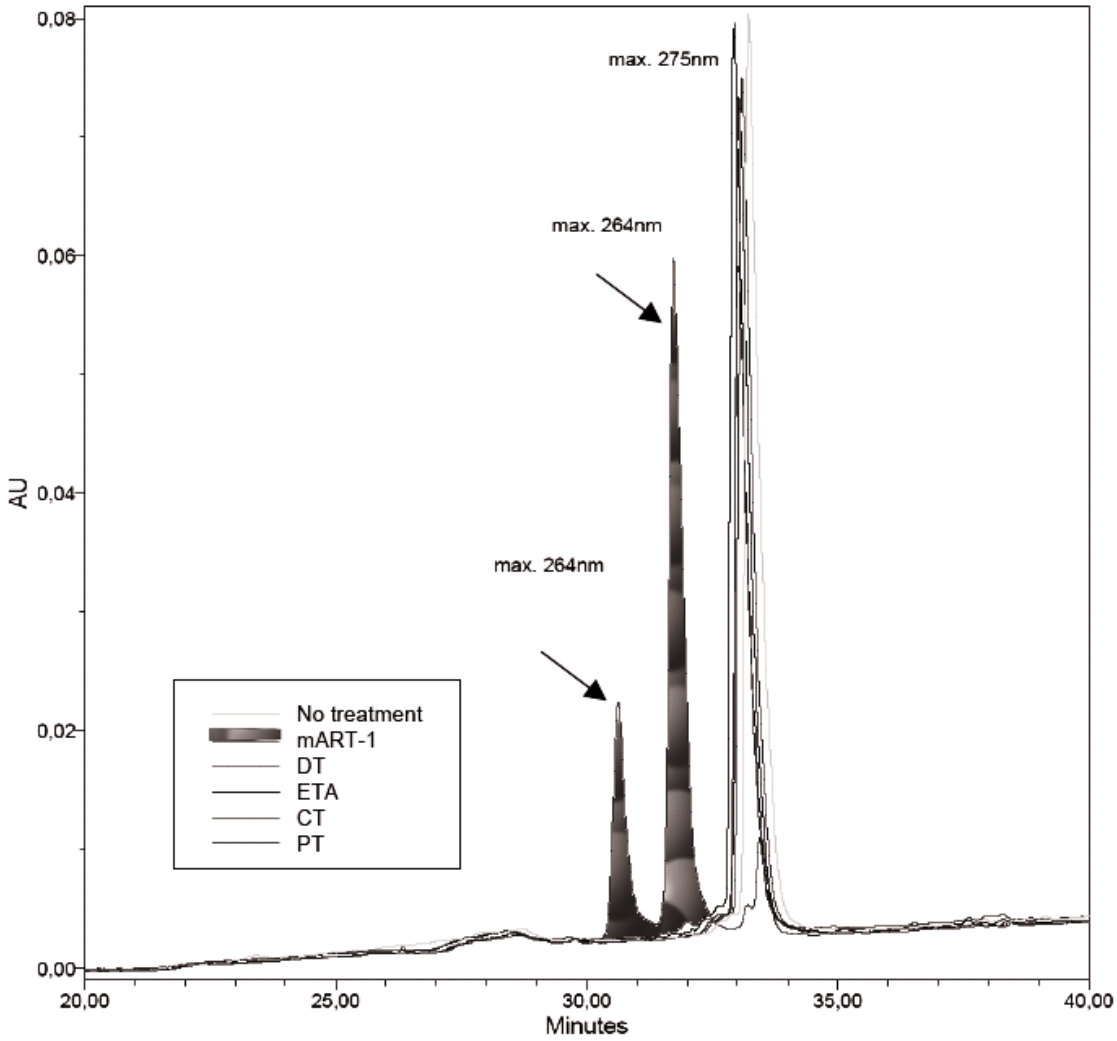

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Fig. 2

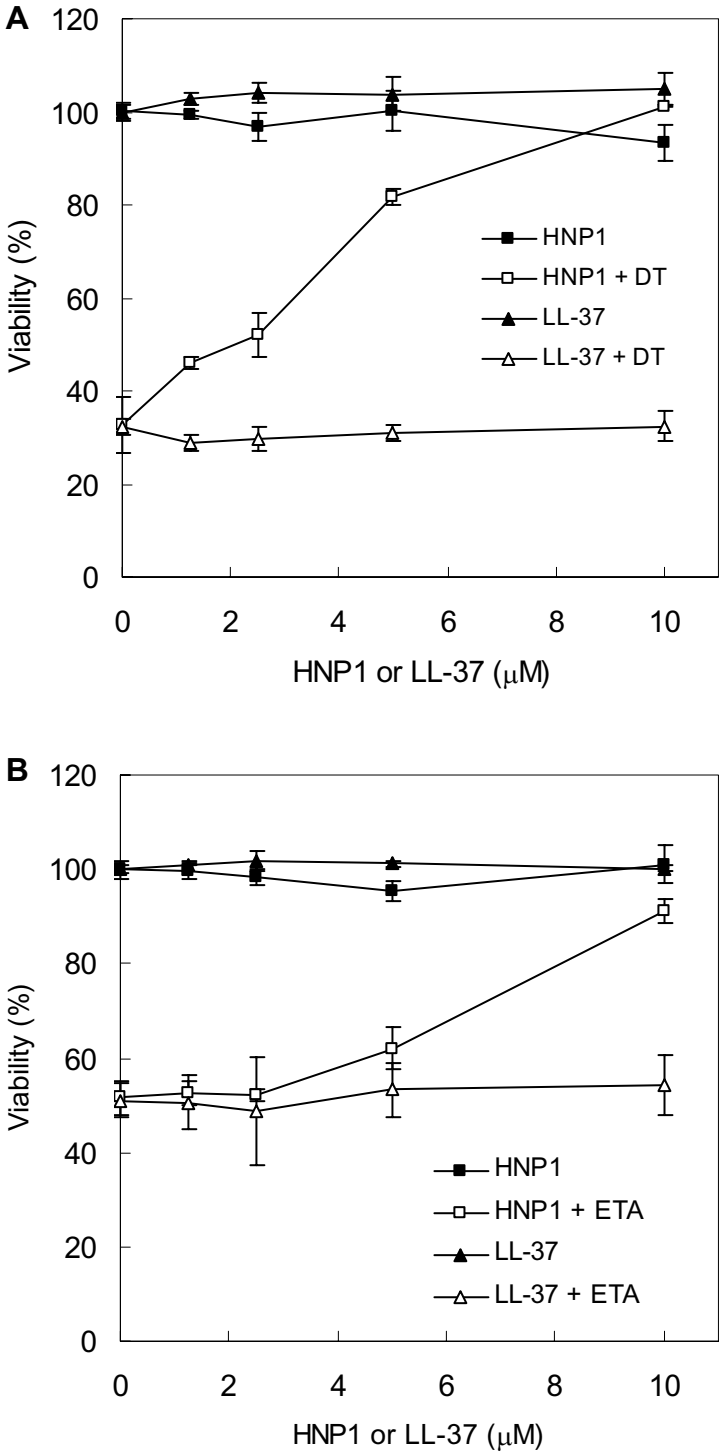

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Fig. 3

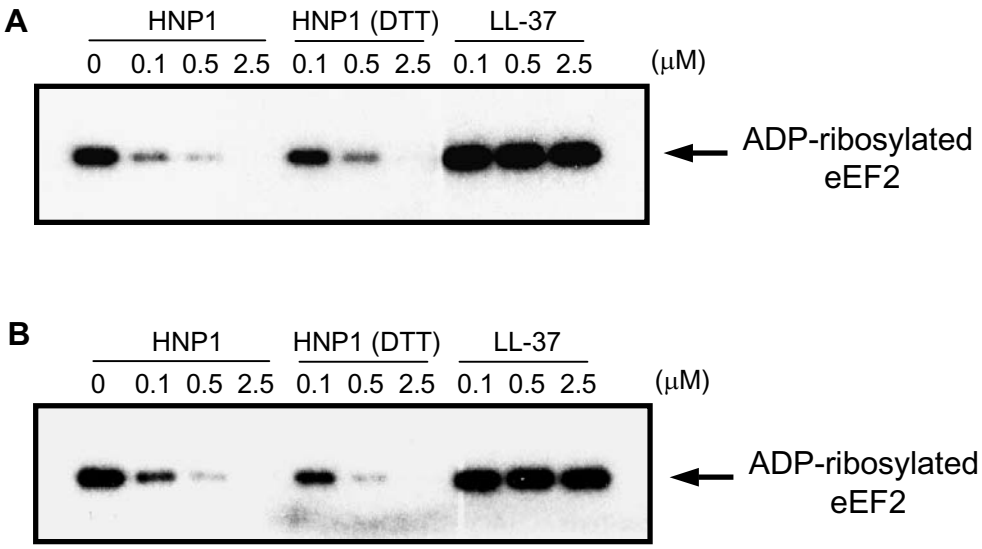

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Fig. 4

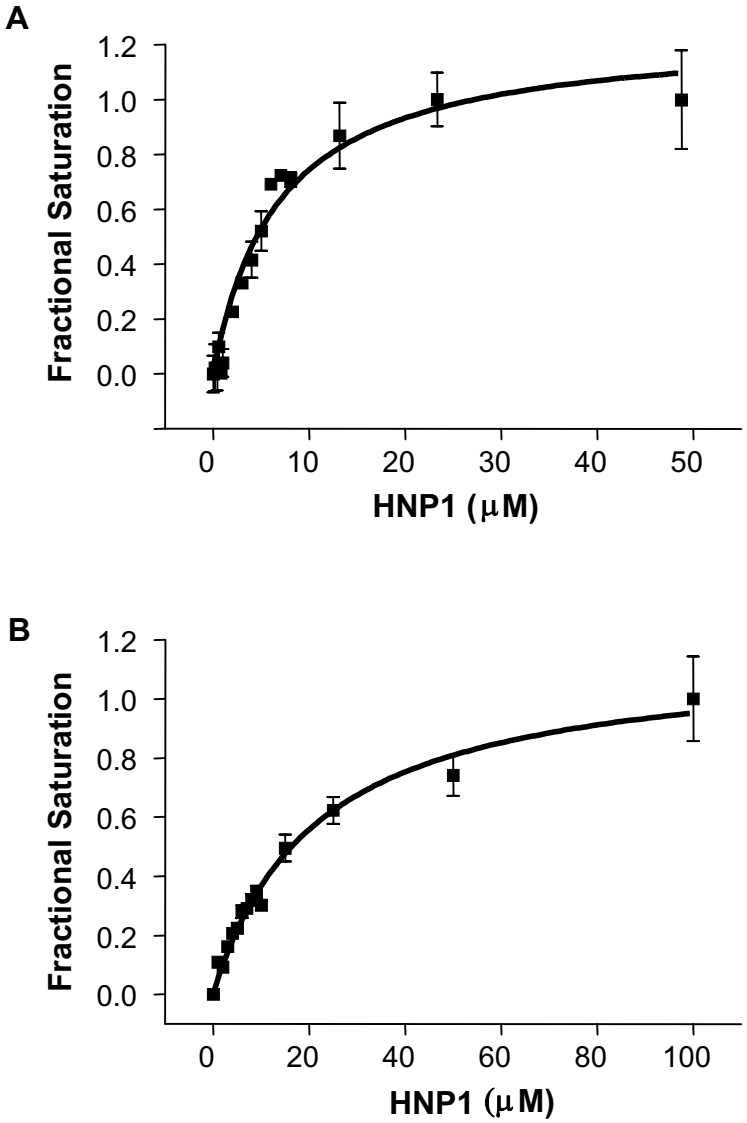

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Fig. 5

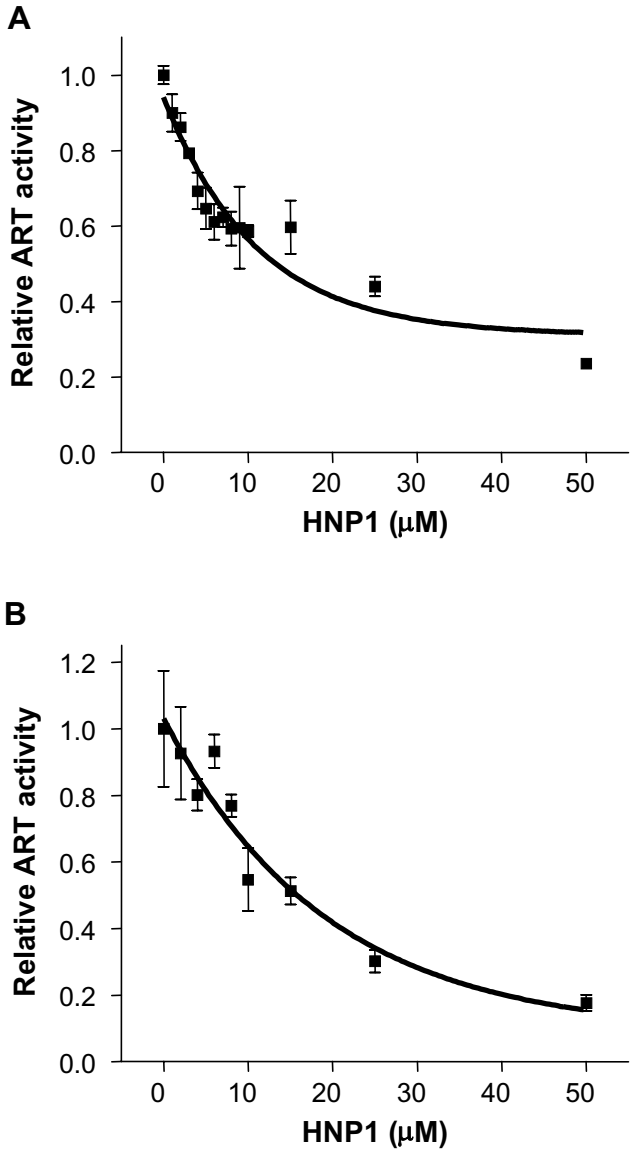

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Fig. 6

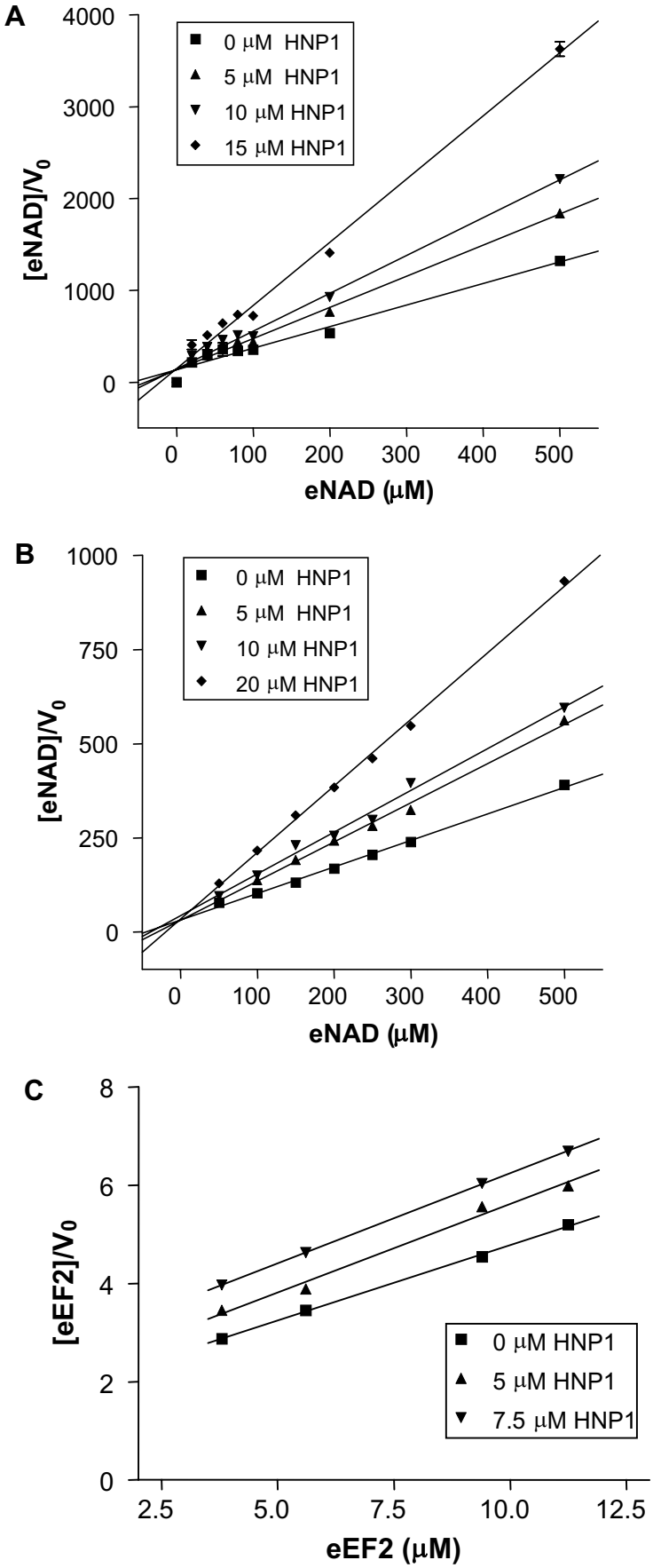

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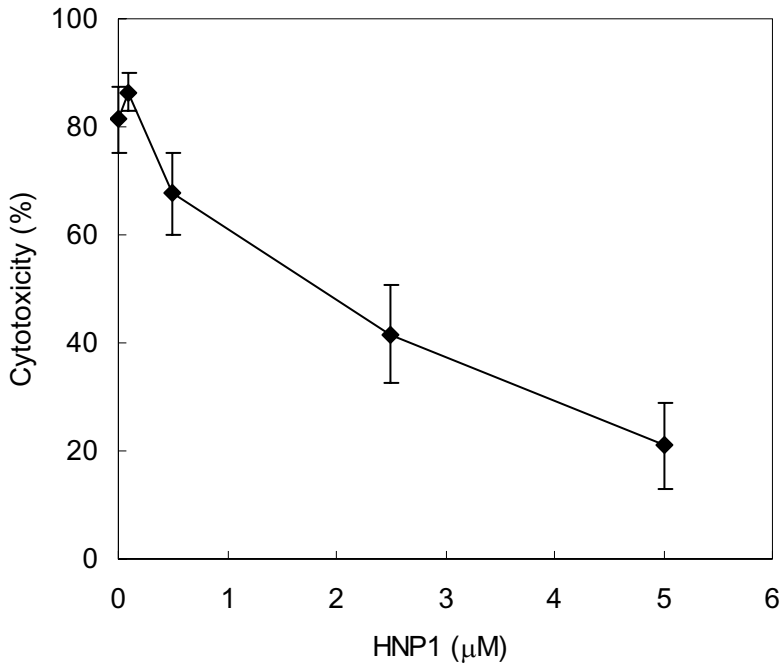
Fig. 7



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