Kinetin riboside and its ProTides activate the Parkinson’s disease associated PTEN-induced putative kinase 1 (PINK1) independent of mitochondrial depolarisation

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ABSTRACT: Since loss of function mutations of PINK1 lead to early-onset Parkinson’s disease, there has been growing interest in the discovery of small molecules that amplify the kinase activity of PINK1. We herein report the design, synthesis, serum stability and hydrolysis of four kinetin riboside ProTides. These ProTides, along with kinetin riboside, activated PINK1 in cells independent of mitochondrial depolarization. This highlights the potential of modified nucleosides and their phosphate prodrugs as treatments for neurodegenerative diseases.

Parkinson’s disease (PD) is the second most common neurodegenerative disease in the world.1 It affects around 130,000 people in the UK and over 1 million people in the USA. Considering that current PD therapies and medical interventions are limited only to addressing the symptoms of this disease2 coupled with a general rise in lifespan, the rate of PD-incidence in the future is likely to increase significantly. This highlights the need for new and specific PD treatments. As part of our efforts into discovering novel PD therapeutics, we focused on PINK1 (PTEN-induced kinase 1), a protein kinase mutated in some patients with early-onset PD.3

PINK1 is a mitochondrial Serine/Threonine protein kinase that possesses a unique N-terminal mitochondrial targeting sequence, a transmembrane domain and three insertional loops within its catalytic kinase domain.4 Following inner mitochondrial membrane depolarization, it becomes stabilized on the outer mitochondrial membrane (OMM) where it phosphorylates the E3 ubiquitin ligase Parkin at Serine 65 (Ser65) on its N-terminal ubiquitin-like domain. Such phosphorylation activates Parkin, which is also mutated in early-onset PD,5 leading to the ubiquitylation of a series of its substrates on the OMM that act as a signal for the degradation of mitochondria by autophagy (mitophagy).6

In PD, the majority of PINK1 mutations are located within its kinase domain and consequently affect its catalytic activity.7,8 This confirms that the kinase activity of PINK1 is critical to the prevention of neurodegeneration. This notion has been verified in Drosophila models of PINK1 in which kinase-inactive versions of PINK1 failed to rescue neurodegeneration compared to the wild-type gene.9 Hence, the activation of PINK1 emerged as a useful strategy to induce and maintain neuroprotective effects, an approach that would be useful in treating PD.

To date, reported efforts into the discovery of small molecules that activate PINK1 led to the identification of N6-furfuryladenine, termed kinetin (1, Figure 1),10 which is undergoing clinical trials for the treatment of Familial Dysautonomia and the prevention from skin photodamage.11 In cells, pronounced activation of PINK1 by kinetin was only observed following co-incubation with the mitochondria-depolarizing agent CCCP (carbonyl cyanide m-chlorophenyl hydrazine),3 which in itself activates PINK1 in cells.12 Studies into the mechanism by which PINK1 is activated by kinetin revealed that kinetin was converted intracellularly in four consecutive metabolic
steps to the active metabolite kinetin riboside (KR) triphosphate 4, which acts as a PINK1 ATP-neosubstrate (Figure 1).20

Given that the cellular activation of synthetic nucleobases and their nucleoside derivatives may be of limited efficiency as compared to natural nucleobases and nucleosides, we explored the direct use of the KR monophosphate intermediate (3) as an activator of PINK1. Using this metabolite, 3, instead of kinetin to activate PINK1 in cells would bypass two important activation steps, glycosylation and the first phosphorylation step, that kinetin must undergo consecutively. This suggests that KR monophosphate would be a more potent activator of PINK1 than kinetin. As nucleoside monophosphates often have poor in vivo stability and inefficient cellular uptake, we employed the ProTide prodrug technology3 to deliver KR monophosphate into cells. This prodrug technology has inspired the discovery of two FDA-approved (antiviral) nucleotide monophosphate and monophosphonate drugs with many more undergoing clinical trials.14

The synthesis of KR ProTides started by making kinetin riboside in a single step from 6-chloropurine riboside (10) as reported (Figure 2).15 This involved refluxing 6-chloropurine riboside with furfurylamine in ethanol in the presence of triethylamine. The pure product was subsequently coupled16 with the appropriate phosphorochloridate (6-9) in the presence of T BuMgCl or NMI as a base to afford the desired KR ProTides (11-14).

In the design of these ProTides, the amino acid of the ProTides was fixed as L-alanine since this historically17 has given the optimum biological activity and is processed well by enzymes during the metabolism of the ProTides in vivo. A small selection of ester motifs was used in this study [methyl (Me), isopropyl (iPr), tert-butyl (tBu) and benzyl (Bn)] to probe the influence of these moieties on the ProTides' biological activity.

Since these ProTides are prodrugs aimed at delivering KR monophosphate, we initially explored the hydrolysis of the phosphate masking groups to release the naked KR monophosphate. The intracellular metabolism18 of ProTides is known to be triggered by esterase enzymes, such as cathepsin A,19 which cleave off the ester motif (Figure 3). The generated carboxylate group (15) undergoes a nucleophilic attack on the phosphate group resulting in the loss of the phenyl group and the formation of an unstable 5-membered heterocyclic ring (16). A water molecule subsequently attacks the phosphate group to open this ring and generate metabolite 17. Finally, a phosphoramidase-type enzyme, e.g. Hint-a,20-22 hydrolysates the P-N bond of metabolite 17 leading to the release of the KR monophosphate.

To probe the hydrolysis of KR ProTides to release KR monophosphate, we followed the hydrolysis of the KR ProTide 14 by 31P-NMR in the presence of recombinant cathepsin A (Figure 4A).16 We chose KR ProTide 14 as it has an L-alanine isopropyl moiety akin to the two ProTides approved for use in the clinics, e.g. Sofosbuvir20 and Tenofovir alafenamide, which both bear the same L-alanine isopropyl ester moiety.17 Before the addition of the enzyme, ProTide 14 (in d6-acetone) showed two peaks (δP 3.58 and 3.88) corresponding to its two diastereoisomers. After ~15 minutes incubation with cathepsin A in TRIZMA buffer, two peaks at δP 3.7 and 4.1 appeared, which correspond to the parent ProTide diastereoisomers under the d6-acetone/TRIZMA buffer. After ca. 2 h, a new peak at δP 6.81 started appearing. This corresponds to metabolite 17 in agreement with previous reports.16 Following 12 h, almost all of ProTide 14 was converted to metabolite 17 and post 48 h incubation a new peak, δP ~ 0.11, appeared, a typical 31P-NMR shift of nucleoside monophosphates.

Figure 1. Chemical structure of kinetin (1) and its metabolism in cells to generate the active substrate kinetin riboside triphosphate (4).

Figure 2. Synthesis of kinetin riboside and its ProTides. Reagents and conditions: (i) POCl3, TEA, Et2O, -78 °C; (ii) L-alanine ester hydrochloride, TEA, DCM, -78 °C; (iii) Furfurylamine, TEA, EtOH, N2, 77 °C; (iv) T BuMgCl or NMI, DCM, N2, rt.

Figure 3. Postulated mechanism of in vivo metabolism of ProTides to release nucleoside analogue monophosphates.
Indeed, negative ion electrospray ionization mass spec analysis of the sample showed that this new peak had a mass of 426.1 g/mol, which matches the mass of KR monophosphate (MW = 427.31 g/mol) (Supplementary figure S1). Although in this sample, there was no phosphoramidase-type enzyme, e.g. Hint-1, that cleaves the P-N bond of intermediate 17 to generate the monophosphate species, it appears that the P-N bond of metabolite 17 was unstable under the assay condition after > 48 h incubation. Together, the data indicated that incubation of a KR ProTide with cathepsin A triggered its hydrolysis to the major metabolite 17 with a trace of KR monophosphate.

Subsequently, we examined the stability of KR ProTides in human and mouse serum in situ. For this, we incubated KR ProTide 14 in either human (Figure 4C) or mouse serum (Supplementary figure S2) at 37 °C and followed the sample by 31P NMR. At t = 0, two close 31P NMR signals, δ 4.0 and 4.5 ppm corresponding to the two diastereoisomers of KR ProTide 14 were present. The time-course 31P NMR revealed that no new peaks appeared that may correspond to new metabolites. Indeed, following ca. 11 h incubation in human and mouse serum there was no changes in the 31P NMR signals that correspond to the KR ProTide indicating its stability in these environments. Additionally, we studied the stability of KR ProTides in acidic environment. For this, we incubated ProTide 14 in acidic buffer, pH = 1, and monitored it by 31P NMR over 12 h. The data showed that the ProTide was completely stable in this acidic environment, pH = 1, since the phospho peaks corresponding to ProTide 14 persisted throughout the 12 h period studied (Supplementary figure S3).

Once the hydrolysis and stability of KR ProTides were established, we then investigated their ability to activate PINK1 in cells. Briefly, HEK293 Flp-In TReX HEK293 cells stably expressing wild-type PINK1 were co-transfected with untagged wild type Parkin. Upon activation, PINK1 directly phosphorylates Parkin at Ser65,17 and this phosphorylation site was used as a read out for the activity of PINK1 in cells. Initially, the cells were treated with 50 µM of kinetin, KR or KR ProTides (11-14) for 24 h (Fig. 5).

Interestingly three out of the four KR ProTides showed activation of PINK1, as judged by P65 phosphorylation in the absence of CCCP treatment. KR ProTide 13 exhibited the most significant activation followed by ProTides 14 and 11. Notably, KR also showed significant activation of PINK1 while treatment with kinetin did not lead to noticeable PINK1 activation in the absence of CCCP.

The activation of PINK1 by KR ProTides indicates that they ProTides were metabolized to release KR monophosphate.
which was then further phosphorylated to the active triphosphate counterpart to act as a PINK1 ATP-neosubstrate. This possibility is supported by the lack of PINK1 activation with KR ProTide 12, which has the tBu ester motif that is known to be poorly metabolized in vivo by esterases as compared to ProTides with Me, iPr and Bn esters. In fact, in vitro cathepsin A hydrolysis of KR ProTide 12 was very slow following 12 h incubation with the esterase enzyme cathepsin A as ca. 50% of the parent ProTide remained intact (Supplementary figure S4) in contrast to ProTide 14, which was rapidly hydrolyzed (Figure 5A). The fact that KR showed comparable PINK1 activation to KR ProTides suggests that the first phosphorylation step by which KR is converted into its monophosphate species, and which is by-passed by the ProTides, is not the rate limiting step in its activation. This is seen with other therapeutic nucleosides such as lamivudine and zidovudine for which the first phosphorylation step is not the rate-limiting in their activation, the second or the third phosphorylation steps.

Since the activation of PINK1 by KR and its ProTides was determined after 24 h incubation (Figure 5), we next determined the time-dependent activation of PINK1 in cells by the most potent KR ProTide activator of PINK1, 13. Under similar conditions, we treated cells with ProTide 13 for 3, 6, 12, 24 and 48 h (Figure 6). The data shows that KR ProTide 13 activated PINK1 in a time-dependent manner with the most prominent activation observed after 24 h treatment. The activation, however, was not as significant as achieved with CCCP and no phosphorylation of Parkin was detected in cells expressing the Parkin S65A mutant as expected.

Figure 6. Time-dependent activation of PINK1 by KR ProTide 13 in Flp-In TRex HEK293 cells stably expressing PINK1 transfected with wild-type. Experiment was performed as in Figure 5.

In conclusion, we herein described the first application of the powerful ProTide phosphate prodrug technology to elaborate nucleoside-based molecules that activate PINK1 in cells. Of the four KR ProTides synthesised and studied in this work, three KR ProTides showed activation of the kinase activity of PINK1 with KR ProTide 13 exhibiting the highest potency. Uniquely, this activation was independent of CCCP, a mitochondrial depolarizing agent, which has been used previously in identifying kinetin as an activator of PINK1. In view of the ability of these KR ProTides to activate PINK1, it may be promising to optimize these further as potential neuroprotective agents for PD. Such endeavours are supported by the favorable human and mouse serum stability profiles of these ProTides in addition to their encouraging stability in acidic environments. Notably, our approach of using the ProTide technology in developing protein kinase activators has the potential to be expanded to the discovery of ATP-neosubstrates for other protein kinases beyond PINK1 and neurodegeneration.

EXPERIMENTAL SECTION

General information. Dichloromethane, diethyl ether, methanol and toluene were dried in-house using a Pure Solv-MD Solvent Purification System. All the other solvents were used as received from commercial suppliers. All of the other reagents used in the synthesis were purchased from Sigma-Aldrich except L-alanine isopropyl ester. All reactions were carried out under an argon atmosphere. Reactions were monitored with analytical TLC on Silica Gel 60–F254 pre-coated aluminum plates and visualized under UV (254 nm) and/or with 31P NMR spectra. Column chromatography was performed on silica gel (35–70 µm). NMR data were recorded either on a Bruker AV300, AVIII300, AV400, AVIII400 or DRX500 spectrometers in the deuterated solvents indicated and the spectra were calibrated on residual solvent peaks. Chemical shifts (δ) are quoted in ppm and J values are quoted in Hz. In reporting spectral data, the following abbreviations were used: s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets) and m (multiplet). HPLC was carried out on a Dionex summit P580 quaternary low pressure gradient pump with a built-in vacuum degasser using a Summit UVD 170s UV/Vis multichannel detector. Solvents were used as HPLC grade. Chromatone software was used to visualize and process the obtained chromatograms. Analytical separations used a flow rate of 1 mL/min, and semi-preparative used a flow rate of 3 mL/min and preparative used a flow rate of 20 mL/min. All tested compounds had a purity of 95% as shown by HPLC or elemental analysis (see Supporting Information).

Kinetin Riboside (4). Furfurylamine (0.54 mL, 6.2 mmol) and Et3N were added dropwise to a suspension of 6-chloropurine riboside6 10 (600 mg, 2.1 mmol) in EtOH (30mL). This was refluxed for 18 h at 60 °C. The resulting yellow solution was evaporated under reduced pressure to yield the crude mixture as a cream paste. This was washed with Et2O (3 x 25 mL) and filtered to give the product as a white crystalline solid (679 mg, 93%); 1H NMR (400 MHz, MeOD) 8.26 (2H, s, H2/H-8), 7.43 (1H, dd, J = 1.9, 0.9 Hz, CH2/Ar-O-H), 6.34 (1H, dd, J = 3.2, 1.8 Hz, CH2/Ar-O-H), 6.31 (1H, dd, J = 3.2, 0.9 Hz, CH2/Ar-O-H), 5.95 (1H, dd, J = 6.4, 1.8 Hz, 1'-H), 4.80 (2H, s, NH2/CH); 4.74 (1H, dd, J = 6.4, 5.1 Hz, 2'-H2), 4.31 (1H, dd, J = 5.1, 2.5 Hz, 3'-H), 4.16 (1H, q, J = 2.5 Hz, 4'-H), 3.81 (2H, q, J = 6.4, 1.8 Hz, 5'-H); 13C NMR (100 MHz, MeOD) 152.0 (C-8/C-2), 151.6 (CH2/Ar-O-C), 149.6, 147.8 (ArN-C), 142.0 (CH2/Ar-O-C), 140.3 (C-8/C-2), 119.9 (ArN-C), 110.0 (CH2/Ar-O-C), 106.9 (CH2/Ar-O-C), 89.9 (1'-C), 86.8 (4'-C), 74.0 (2'-C), 71.3 (5'-C), 62.1 (5'-C), 36.7 (NH/CH); MS-ESI (m/z): C10H8N2O2 [M + Na]+ 370.1.

Phenyl-(methoxy-L-alaninyl)-phosphorochloridate (6). L-alanine methyl ester hydrochloride (400 mg, 2.9 mmol) was dissolved in anhydrous CH2Cl2 (20 mL) under an inert atmosphere. Following this, phenyl phosphorochloridate (0.53 mL, 2.9 mmol) was added dropwise over 15 min. Et3N (0.77 mL, 5.8 mmol) was then added at -78 °C and the mixture was stirred for 30 min. The solution was then allowed to warm to room temperature over 2 h. Solvent was then removed under reduced pressure and the remaining white precipitate suspended in anhydrous Et2O (50 mL). This was filtered and the filtrate
evaporated under reduced pressure, yielding the crude product as a light brown oil. Purification via flash column chromatography gave the pure product as a colourless oil (729 mg, 9%); ([20 mg, 1.8 mol %]) ESI (m/z): Calc. for 

Phenyl-(tert-butyloxy-L-alanyl)-phosphorochloridate (7). Prepared as described for compound 6 using L-alanine tert-butyl ester hydrochloride (400 mg, 2.20 mmol), phenyl phosphorochloridate (0.30 mL, 2.20 mmol) and Et,N (0.60 mL, 4.4 mmol). Product 7 was observed as a pale yellow oil, which was used in sequential steps without further purification (803 mg, 11%).

Phenyl-(benzylxy-L-alanyl)-phosphorochloridate (8). Prepared as described for compound 6 using L-alanine benzyl ester hydrochloride (500 mg, 2.32 mmol), phenyl phosphorochloridate (0.35 mL, 2.32 mmol) and Et,N (0.63 mL, 4.64 mmol). Product 8 was observed as a pale yellow oil (505 mg, 95%).

Phenyl-(isopropoxy-L-alanyl)-phosphorochloridate (9). Prepared as described for compound 6 using L-alanine isopropyl ester hydrochloride (700 mg, 4.18 mmol), phenyl phosphorochloridate (0.62 mL, 4.18 mmol) and Et,N (1.13 mL, 8.36 mmol). Product 9 was observed as a yellow oil (185 mg, 93%).

Phenyl-(methoxy-L-alanyl) kinetin riboside phosphoribosidase (11). KR 2 (200 mg, 0.60 mmol) was suspended in anhydrous THF (15 mL) under an inert atmosphere. To this, tBuMgCl (0.09 mL, 0.68 mmol) was then added dropwise over 15 min. The resulting solution was stirred for 10 min and following this, 6 (241 mg, 0.86 mmol) in anhydrous THF (1.5 mL) was then added dropwise over 10 min. The mixture was left to stir for 18 h. MeOH (2 mL) was then added to quench the reaction before being removed under reduced pressure to leave the crude product as a pale yellow oil. This was purified via flash column chromatography and then preparative TLC to yield the final product as a white solid (34 mg, 10%); (Eluent 3:079 to 5.95 MeOH/CHCl3); P3N NMR (120 MHz, CDCl3) δ 8.27 (s, H, Ar-CH3), 8.23 (s, H, Ar-CH3), 7.43 (s, H, CHArO-H), 7.30 (2H, t, J = 7.9 Hz, Ph-H), 7.22-7.12 (3H, m, Ph-H), 6.34 (Hd, d = 3.4, 1.8 Hz, CHArO-H), 6.32 (Hd, d = 2.8 Hz, CHArO-H), 6.04 (Hd, d = 5.0 Hz, 1'-H), 4.80 (2H, br s, NHPh), 4.70-4.60 (Hd, m, 2Hl), 4.46-4.30 (3H, m, 3'H/5'H), 4.29-4.23 (2H, H, 4'-H), 3.95-3.77 (2H, m, CH2Ph), 3.61 (3H, s, OCH3), 3.10-2.77 (2H, m, CH2Ph), 2.54 (3H, s, OCH3), 2.14 (3H, s, OCH3).

Phenyl-(methoxy-L-alanyl) kinetin riboside phosphoribosidase (14). Prepared as described for compound 11 using 2 (250 mg, 0.72 mmol), NMI (0.29 mL, 3.66 mmol) and 9 (600 mg, 2.16 mmol). The crude product was purified via flash column chromatography to yield the final product as a white solid (85 mg, 42%); (Eluent 3:079 to 5.95 MeOH/CHCl3); P3N NMR (120 MHz, CDCl3) δ 8.02, 8.28; 8.89 (s, H, Ar-CH3), 7.98, 7.94 (s, s, Ar-CH3), 7.30 (Hd, d = 2.5 Hz, CHArO-H), 7.15 (2H, t, J = 7.7 Hz, Ph-H), 7.07 (2H, d, J = 7.7 Hz, Ph-H), 7.00 (Hd, d = 5.0 Hz, 1'-H), 5.98 (2H, br s, NHPh), 4.78 (2H, br s, NHPh), 4.57-4.25 (3H, m, 3'H/5'H), 4.09-3.81 (2H, m, CH2Ph), 3.14 (3H, s, OCH3), 2.54 (3H, s, OCH3), 2.13 (3H, s, OCH3).

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(ArO-CH), 88.9 (1'-C), 83.1 (4'-C), 74.5 (2'-C), 70.4 (3'-C) 68.9 (OCH2Pr), 65.8 (5'-C), 50.0 (NHCH2CH2), 37.2 (NHCH2Ar), 21.4 (OCH2Pr), 20.5 (NHCH2CH2); HRMS-ESI (m/z): Calc. for C34H33NO6P [M + H]+: 617.2123, found: 617.2144; HPLC CH3CN:H2O 0.100 to 1.000 in 30 min, λ = 254 nm, tR 16.51, 16.69 min.

**Cathepsin A Assay.** Procedure adapted from Mehellou et al., 2010. ProTide 14 (5.0 mg) was dissolved in acetone-d6 (0.15 mL), followed by addition of Trizma buffer (0.30 mL, pH 7.4). After recording a control 31P NMR spectrum containing ProTide in d6-acetone and buffer, defrosted cathepsin A (0.1 mg dissolved in 0.15 mL of Trizma buffer), was added to the mixture. A 31P NMR was then run immediately after the addition and then at even time intervals over 11 h. The sample was then analysed by 31P NMR after 24 and 48 h. All 31P NMR spectra recorded at 22 °C (21).

**Serum stability.** Procedure adapted from Slusarczyk M. et al. 2014.17 ProTide 14 (5.0 mg) was dissolved in DMSO-d6 (0.10 mL) and D2O (0.15 mL). All 31P NMR spectra were recorded at 37 °C. Two control spectra were recorded, one containing ProTide 14 (5.0 mg) in DMSO-d6 (0.10 mL) and D2O (0.15 mL) and the other containing defrosted human serum (0.3 mL), DMSO-d6 (0.10 mL) and D2O (0.15 mL). Following this, a previously defrosted serum (human or mouse) (0.30 mL) was added to the NMR tube and a spectrum immediately run. Spectra were recorded at 30 min after the addition and then at even time intervals over 11 h.

**Acid Stability.** Procedure adapted from Slusarczyk M. et al. 2014.17 To ProTide 12 (5 mg) in methanol-d4 (0.25 mL) was added acidic buffer, pH 1 (prepared from equal parts of 0.2 M HCl and 0.2 M KCl). The sample was then subjected to 31P NMR experiments at 37 °C and the spectra were recorded every 20 min over 12 h.

**Docking studies.** PC windows 7 with Intel Core i7-4790 3.6GHz microprocessor, 16 GB RAM and 64 Bit operating system was used to execute the molecular computational studies. Docking was performed applying the molecular modelling modules namely Omega2, FRED and VIDA provided by OpenEye Scientific Software (http://www.eyesopen.com). The three-dimensional structure of the co-crystallized AMP with the Human HINT1 was retrieved from the Protein Data Bank (PDB ID: 1KPF)48 and the active site was subsequently identified on the basis of the bound ligand. Multiple conformers for metabolite 17 were generated by Omega2 using the default settings.57 FRED (fast rigid exhaustive docking) implements a rigid docking approach to fit these conformers into the pre-defined binding site and rank the poses by scoring functions.28,29 The VIDA module was then used to visualize and inspect the docked poses within the receptor’s active site as well as to identify the main interacting residues.

**Cell studies.** Flp-In T-Rex HEK293 cells stably expressing PINK1-FLAG wild-type were generated previously.53 Cells were maintained in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10 % (v/v) foetal bovine serum, 2 mM L-glutamine, 100 μM penicillin and 0.1 mg/ml streptomycin, plus 15 mg/l plasticid and 100 μg/ml hygromycin at 37 °C under a 5% CO2 atmosphere. On day 0, cells were seeded in DMEM. On day 1, Cells were transiently transfected with wild type or S65A Parkin using polyethyleneimine (Polysciences) according to the manufacturer’s instruction. On day 2, PINK1-FLAG overexpression was induced by adding 0.1 μg/ml doxycycline in DMEM for 24 h before treating cells with compounds or mitochondrial uncoupler, CCCP, as indicated in the figure legends. All compounds were dissolved in DMSO.

**Sample preparation and immunoblotting.** This was carried out as we reported previously.63 Lysis buffer used: 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1 % (v/v) Triton, 1 mM sodium orthovanadate, 10 mM sodium glycerophosphate, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 0.25 M sucrose, 0.1 % (v/v) 2-mercaptoethanol, 1 mM benzamidine, 0.1 mM PMSF and protease inhibitor cocktail (Roche). Antibodies used: mouse monoclonal anti-PINK1 antibody (human PINK1 residues 125-539) was raised by Dundee Cell Products, anti-vinculin and anti-GAPDH antibodies were obtained from Cell Signaling Technology, anti-Parkin phospho-Serine 65 rabbit monoclonal antibody was raised by Epitomics in collaboration with the Michael J Fox Foundation for Research, anti-Parkin mouse monoclonal antibody was obtained from Santa Cruz.

**ASSOCIATED CONTENT**

Supporting Information. 1H NMR and 13C NMR spectra, HPLC data and the mass spectra of the cathepsin A sample. This material is available free of charge via the Internet at http://pubs.acs.org.

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**Notes**

The authors declare no conflict interests.

**Author Contributions**

The manuscript was written by YM and all the authors provided comments and gave approval to the final version of the manuscript. These authors contributed equally.

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**ABBREVIATIONS**

CCCP, carbonyl cyanide m-chlorophenyl hydradrazine; DCM, dichloromethane; Et2O, diethyl ether; Et3N, triethylamine; EtOH, ethanol; KR, kinetin riboside; NMI, N-methylimidazole; OMM, outer mitochondrial membrane; PD, Parkinson’s disease; PINK1, PTEN-induced putative kinase 1; POCl3, phosphorous oxychloride; PTEN, phosphatase and tensin homolog; RT: room temperature; tBuMgCl, tert-butyl magnesium chloride; TEA, triethylamine; Trizma: (tris(hydroxymethyl)aminomethane).

**REFERENCES**


Supporting Information

Kinetin riboside and its ProTides activate the Parkinson's disease associated PTEN-induced putative kinase 1 (PINK1) independent of mitochondrial depolarisation


I. Supplementary Figues

II. $^1$H, $^{13}$C and $^{31}$P NMR and HPLC Spectra

2. Compound 6 ..........................................................7-8.
5. Compound 9 ..........................................................11-12.

III. HPLC spectrum of 14 ..........................................................19.
I. Supplementary Figures

Supplementary figure S1. ESI Mass spec analysis of sample generated from the in vitro cathepsin A-mediated hydrolysis of KR ProTide 14. MW 426.1 g/mol corresponds to metabolite 17.
Supplementary figure S2. Stability of KR ProTide 14 in mouse serum over 12 h as monitored by $^{31}$P NMR. Peaks under rectangle 1 correspond the diastereoisomers of KR ProTide 14 while that under 2 is from the mouse serum.
Supplementary figure S3. Stability of ProTide 14 in acidic environment monitored by $^{31}$P NMR. The two peaks highlighted in the orange rectangle ($\delta_p$ 3.02 and 2.86) correspond to the two diastereoisomers of ProTide 14 in MeOD. Acidic buffer, pH = 1, consisted of 0.2 M HCl and 0.2 M KCl. The experiment was run at 37 °C and the sample was analysed by $^{31}$P NMR every 30 minutes for 12 h. A selection of the data at the indicated times is shown in the figure.
Supplementary figure S4. $^{31}$P-NMR of cathepsin A mediated in vitro degradation of KR ProTide 12. Figure showing KR ProTide 12 alone and then the sample after the addition of cathepsin A and reading at different time points; 10 and 30 min, 1, 4 and 11 h.
II. $^1$H, $^{13}$C and $^{31}$P NMR and HPLC Spectra
Analytical HPLC of LO-28 (ProTide 14)

Sample Name: LO28  Injection Volume: 10.0
Vial Number: 1_3  Channel: UV_VIS_3
Sample Type: unknown  Wavelength: 254
Control Program: MeCN 30min gradient  Bandwidth: 8
Quantif. Method: General Method  Dilution Factor: 1.0000
Recording Time: 1/3/2016 17:37  Sample Weight: 1.0000
Run Time (min): 40.00  Sample Amount: 1.0000

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