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Small molecule inhibitors of arginyltransferase regulate arginylation-dependent protein degradation, cell motility, and angiogenesis

Sougata Saha [a,b], Junling Wang [a], Brian Buckley [c], Qingqing Wang [d], Brenda Lilly [d], Mikhail Chernov [c], Anna Kashina [a,*]

[a] Department of Animal Biology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA 19104, United States
[b] Department of Molecular Biology and Biotechnology, Tezpur University, Napaam 784028, India
[c] Roswell Park Cancer Institute, Buffalo, NY 14263, United States
[d] Nationwide Children's Research Institute, Department of Pediatrics, The Ohio State University, Columbus, OH 43205, United States

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ABSTRACT

Posttranslational arginylation mediated by arginyltransferase (ATE1) is an emerging major regulator of embryogenesis and cell physiology. Impairments of ATE1 are implicated in congenital heart defects, obesity, cancer, and neurodegeneration making this enzyme an important therapeutic target, whose potential has been virtually unexplored. Here we report the development of a biochemical assay for identification of small molecule inhibitors of ATE1 and application of this assay to screen a library of 3280 compounds. Our screen identified two compounds which specifically affect ATE1-regulated processes in vivo, including tannic acid, which has been previously shown to inhibit protein degradation and angiogenesis and to act as a therapeutic agent in heart disease and cancer. Our data suggest that these actions of tannic acid are mediated by its direct effect on ATE1, which regulates protein degradation and angiogenesis in vivo.

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1. Introduction

Posttranslational arginylation is a protein modification of emerging global importance, implicated as a key regulator of embryogenesis and cell physiology. Knockout of arginyltransferase (*Ate1*) causes embryonic lethality in mice with severe defects in cardiovascular development and angiogenesis [1]. A large number of cytoskeleton proteins are arginylated in vivo [2] and arginylation of β-actin is found to be critical for cell motility and the formation of the cell leading edge [3]. Arginylation has also been shown to regulate actin polymer level and the structure of the intracellular actin network [4], and affect cell adhesion, cell migration speeds, and migration-dependent tissue morphogenesis during development [3]. Thus, arginylation plays major roles in cell migratory processes and exerts at least some of its effects through the modulation of the actin cytoskeleton, however the underlying molecular mechanisms are poorly understood.

ATE1 is a highly functionally conserved enzyme in all eukaryotic species, essential for normal development and functioning of mammalian organisms. Impairments in ATE1 regulation have been implicated in such major diseases as congenital heart defects [1], obesity [5], cancer, and neurodegeneration [6,7], making this enzyme a potentially critical target for the development of therapeutics that could modulate these disease conditions and prevent their progression in humans. However, since the molecular properties and structure of this enzyme are not well understood, targeted approaches to modulation of ATE1 activity and functions in vitro and in vivo have never been undertaken before.

Several natural and artificial compounds that influence ATE1 activity in various systems have been identified through the past studies of ATE1-regulated processes, however none of these compounds have high specificity for ATE1 enzyme and most of them have none, or very limited activity in cells. Tri-peptide Glu-Val-Phe can inhibit arginylation by acting as a substrate mimic that saturates ATE1, making it unavailable for arginyl transfer to its natural targets [8], however this peptide acts only at high concentrations and is not very effective in biological assays [8,9]. Bifunctional phenylarsenoxide was shown to inhibit ATE1 through interaction with reactive Cys residues in the critical

Abbreviations: Ate1, arginine transfer enzyme 1 or arginyltransferase 1; RRS, Arg-tRNA synthetase; BSA, bovine serum albumin; RGS4, regulator of G protein signaling 4; VEGF-A165, vascular endothelial growth factor A165; HUVEC, human umbilical vein endothelial cells; TRITC, retramethyl rhodamine isothiocyanate; CXCL12, C-X-C motif ligand 12; ECGS, endothelial cell growth supplement; bFGF, basic fibroblast growth factor.

* Corresponding author at: 3800 Spruce St. 143 Rosenthal, Philadelphia, PA 19104, United States. Tel.: +1 215 746 0895.
E-mail address: akashina@vet.upenn.edu (A. Kashina).

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positions within the molecule [10], however this inhibitor is not only toxic but relatively non-specific, since it exerts its effect similarly on all proteins whose activity is dependent on reactive Cys groups. Heparin, a widely used anticoagulant, inhibits ATE1 reaction in vitro [11], possibly through its action on Arg-tRNA synthetase (RRS) which produces Arg-charged tRNA used for arginyl transfer [12]. Similarly protease inhibitors indirectly inhibit protein arginylation in brain extracts by interfering with the charging of tRNA [13]. Finally, hemin, the Fe^{3+} form of heme, was shown to inhibit ATE1 and promote its degradation in cells through ubiquitin-dependent proteolysis – an indirect effect, likely linked to hemin's action on proteasome, and possibly on RRS [14]. Thus, no natural or artificial compounds are known to date which could specifically modulate ATE1 activity and/or its intracellular functions.

Here we report the development of a chemical assay for identification of small molecule inhibitors of ATE1 and application of this assay to screening of a small molecule library of 3280 known chemicals. Our screen identified four molecules that can specifically inhibit the activity of ATE1, including two compounds which specifically affect ATE1-regulated processes in cells. One of these compounds – tannic acid – has been previously shown to inhibit protein degradation and angiogenesis in cell and mouse models and to act as a therapeutic agent in prevention and treatment of heart disease and cancer. Our data suggest that these actions of tannic acid are mediated by its direct effect on ATE1, which regulates protein degradation and angiogenesis in vivo.

2. Material and methods

2.1. Antibody generation and purification

N-terminally arginylated β actin peptide 'RDDIAALC' was used to raise polyclonal anti-R-β antibody in rabbits. Immunizations and collection of antisera were performed by Sigma Genosys (http://www.sigmaaldrich. com/Brands/Sigma_Genosys.html). Crude antisera was first affinity purified using the immunization peptide immobilized on Aminolink resin (Pierce), and then further purified by immunodepletion with Aminolink-coupled non-arginylated peptide, in which the N-terminal R was replaced with acetylated Asp (ac-D) – 'Ac-DDDIAALC' – a sequence corresponding to the nonarginylated β actin N-terminus in vivo.

2.2. ATE1 assay in microplates and small molecule screen

384-well high binding white plates (Corning) were coated with 1 μg of 'DDIAALVVDNGSGMCK' peptide per well by incubating in 25 μl 23 μM peptide solution in carbonate/bicarbonate buffer (28.6 mM Na_2CO_3/71.4 mM $NaHCO_3$, pH 9.6) at 25 °C for 90 min. After coating, plates were blocked with 5% milk in PBS at 37 °C for 1 h followed by three washes with PBS at room temperature.

For ATE1 assay, 25 μl reaction mix (50 mM HEPES pH 7.5, 25 mM KCl, 15 mM MgCl2, 0.1 mM DTT, 6 mM ATP, 100 nM $tRNA_{Arg}$, 0.5 mM Arginine, 66 nM RRS, and 250 nM ATE1) was added to each well and incubated for 30 min at 37 °C. After the completion of the reaction, plates were washed three times with PBS containing 0.05% Tween 20 (PBST). For detection of arginylated products and measuring the reaction efficiency and ATE1 inhibition, plates were incubated first with anti-R-β antibody (1 h, room temperature), washed three times with PBST, and incubated with HRP-conjugated anti rabbit IgG (1 h, room temperature). After the final incubation plates were washed again 3× with PBST, 25 μl of chemiluminescence substrate (Thermo SuperSignal ELISA Femto, Thermo Fisher) was added to each well and readings were collected between 5 and 15 min of substrate addition. Readings were performed by the Envision 2103 Multilabel Reader (PerkinElmer) equipped with Enhanced Luminescence sensor. For the control experiments shown in Fig. 2B, individual components of the assay were omitted and/or 1% of DMSO was added to the wells, as indicated.

Small molecule screen was performed using this assay system with the following modifications. For the initial screen (SCREEN1), the ATE1 reaction mix was prepared in two parts: (1) a mixture of all the components listed above except arginine, and (2) a separate solution of Arg. Part 1 was added first, followed by the addition of the drug (dissolved in DMSO) delivered by JANUS automated liquid handling system (PerkinElmer) equipped with 384-well pin tool (V&P Scientific, Inc.), at an approximate volume of 30 nl/well. Final concentration of drugs and DMSO in the assay was 14 μM and 0.14% respectively. Arg was added afterwards to start the reaction. In the repeated screen, 8.3 μM of the drugs was used and the compounds that did not inhibit ATE1 reaction at this reduced concentration were discarded as the likely non-specific inhibitors. For the counterscreen (SCREEN 2), the RRS reaction was performed separately by mixing all the components from the ATE1 reaction mix (see above) except ATE1, followed by EtOH precipitation to isolate charged Arg-tRNA as described in [15]. The counterscreen was performed by adding ATE1 to the wells, followed by the addition of the drug, and finally the addition of 3 μM purified Arg-tRNA to start the reaction. The molecules which showed 94% or higher inhibitory activity compared to the positive control were selected for further analysis.

2.3. Cell culture, transfection and drug treatment

Immortalized wild-type (WT) and *Ate1* knockout mouse embryonic fibroblasts [1,3] were grown in DMEM/F10 medium with 10% serum. For RGS4 degradation assays, cells at 60% confluency were transfected with RGS4-His-V5 construct [16] using Lipofectamine reagent (Invitrogen). After 18 h of transfection, cells were split and seeded at 1.25×10^5 cells into individual wells of 24-well plates, and grown for additional 24 h, with or without the addition of the drug (added to the media at the concentrations indicated in Fig. 4B). The entire well contents was then collected for each data point, by resuspending cells directly in 2× SDS loading buffer, and analyzed by Western blots using anti V5 antibody as described in [16]. For wound healing assays, 0.3×10^6 cells were seeded in 35 mm glass bottom dishes (MatTek Corporation) to make confluent monolayers. After 16–18 h, drugs were added to the experimental cultures as indicated in Fig. 5 and control and drug-treated cells were incubated for additional 24 h, followed by scratch wounding and 2 h recovery before performing live imaging or fixing for fluorescence staining. Cell migration speed was measured by time lapse imaging of cell movement into the wound area over 8 h, acquired at the rate of 1 frame per 10 min; distance between the wound edge at the start and end of the movie was divided by the overall acquisition time to obtain the μm/h values shown in Fig. 5B, D.

2.4. Immunofluorescence

Confluent or scarce cells after 24 h of drug treatment were fixed by addition of 4% paraformaldehyde in PBS for 30 min at room temperature, followed by permeabilization by 0.2% Triton X100 in PBS containing 0.2% BSA for 10 min and were blocking with 1% BSA/0.02% Triton X100 in PBS 30 min. Actin filaments were visualized by staining with alexa488-labeled phalloidin.

2.5. Angiogenesis assay

Angiogenesis assay was performed as described [17]. Briefly, 1 ml of collagen/media solution was prepared on ice by adding 340 μl of type I rat tail collagen (BD Biosciences), 76 μl 10× M199

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