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Natural cardenolides suppress coronaviral replication by downregulating JAK1 via a Na\(^+\)/K\(^+\)-ATPase independent proteolysis

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ABSTRACT

An unprecedented biological function of natural cardenolides independent of their membrane target Na\(^+\)/K\(^+\)-ATPase is disclosed. Previously, we reported that cardenolides impart anti-transmissible gastroenteritis coronavirus (anti-TGEV) activity through the targeting of Na\(^+\)/K\(^+\)-ATPase and its associated PI3K_PDK1_RSK2 signaling. Swine testis cells with Na\(^+\)/K\(^+\)-ATPase α1 knocked down exhibited decreased susceptibility to TGEV oncorusivirus (anti-TGEV) activity through the targeting of Na\(^+\)/K\(^+\)-ATPase and its associated PI3K_PDK1_RSK2 signaling. Herein, we further explored a Na\(^+\)/K\(^+\)-ATPase-independent signaling axis induced by natural cardenolides that also afforded significant anti-coronaviral activity for porcine TGEV and human HCoV-OC43. Using pharmacological inhibition and gene silencing techniques, we found that this anti-TGEV or anti-HCoV-OC43 activity was caused by JAK1 proteolysis and mediated through upstream activation of Ndfip1/2 and its effector NEDD4. This study provides novel insights into the pharmacological effects of natural cardenolides, and is expected to inform their future development as antiviral agents.

1. Introduction

Cardenolides and bufadienolides are steroids incorporating a five- or six-membered lactone ring and have a plethora of biological activities. They are best known for their toxicity, which is due to their inhibition of the Na\(^+\)/K\(^+\)-ATPase (or the Na\(^+\)-K\(^+\) pump) involved in the maintenance of ion concentrations in cells and neurotransmission, although they have found clinical utility for the treatment of cardiovascular problems.[1] However, in addition to Na\(^+\)/K\(^+\)-ATPase, there are several other potential pharmacological targets of cardenolides, e.g. steroid receptors.[2,3] Also, ouabain and digoxin are both antagonists of estrogen receptor (ER) that could be useful in the development of breast cancer drugs[1]; ouabagenin, the hydrolysis product of ouabain, is a novel ligand for the liver X receptor (LXR)[4]; and innovative derivatives of cardiotonic steroids (cardenolides and bufadienolides) that target Na\(^+\)/K\(^+\)-ATPase are under development for the optimization of their cardio therapeutic effects[3–6].

Janus kinase 1 (JAK1) is a member of the JAK family of intracellular, nonreceptor tyrosine kinases; essential for signaling certain type I and type II cytokines or interferons[7,8]. JAKs initiate responses to cytokine signaling and immunoregulation as well as inflammation; and JAK1 may contribute to hepatitis C virus (HCV)-mediated pathogenesis via cooperation with HCV nonstructural protein 5A (NS5A) activated signal transducer and activator of transcription 3 (STAT3)[9]. Although JAK/STAT pathway is well-documented to be mainly activated through interferons for antiviral host defenses, viruses have evolved strategies to escape from antiviral host defenses and/or also to exploit activation of non-canonic JAK/STAT pathway to promote viral pathogenesis[10]. In addition to inhibition in viral replication[11], JAK1-signaling inhibition also suppresses tumor progression and metastasis[12], and is currently a therapeutic strategy for inflammatory diseases, e.g. rheumatoid arthritis[13].

Recently, we reported that cardenolides exert potent anti-transmissible gastroenteritis coronavirus (anti-TGEV) activity,[14,15] in part through augmenting a TGEV-induced and Na\(^+\)/K\(^+\)-ATPase-dependent phosphoinositide 3-kinase, 3-phosphoinositide-dependent kinase 1, ribosomal S6 kinase 2 (PI3K-PDK1-RSK2) signaling axis[15]. Furthermore, the PI3K inhibitor LY294002 could restore only part of the TGEV activity inhibited by ouabain[15]; and therefore other signaling axes are posited to co-exist with PI3K-PDK1-RSK2, and co-contribute to the anti-TGEV activity for ouabain. Thus, additional studies are needed to have a comprehensive knowledge for the underlying mechanisms of action of anti-TGEV cardenolides.

Herein, we disclose the results of further investigations into the underlying anti-TGEV mechanisms of natural cardenolides. We found natural cardenolides also downregulated Na\(^+\)/K\(^+\)-ATPase-independent JAK1_STAT1/3 activation to inhibit viral activities of porcine TGEV and human coronavirus OC43 (HCoV-OC43), and confirmed the co-

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existence of the Na⁺/K⁺-ATPase-independent JAK1 diminishment with the augmented activation of Na⁺/K⁺-ATPase-dependent PI3K_PDK1_RSK2 which was reported previously [15]. Both signaling axes of augmented PI3K and downregulated JAK1 contributed to the anti-TGEV activity of natural cardenolides. Finally, we determined that ouabain downregulated JAK1/STATs signaling, at least in part, via NEDD4 E3 ubiquitin protein ligase (NEDD4) family interacting protein 1/2 (Ndfip1/2) and NEDD4 mediated JAK1 proteolysis.  

2. Results  

2.1. Cardenolides diminished JAK1 protein levels and the downstream phosphorylation of STAT3 or STAT1  

Recently, TGEV was reported to activate the JAK_STAT pathway; [16,17] and we reported that the PI3K inhibitor LY294002 restored only part of that TGEV activity inhibited by ouabain, reaching a plateau at high concentrations, whereas ouabain enhanced PI3K_PDK1 activation, was associated with Na⁺/K⁺-ATPase, and contributed to anti-TGEV activity [15]. Accordingly, we sought to determine whether any JAK family kinase is associated with the anti-TGEV activities of ouabain, in addition to the pathways previously screened [15].  

TGEV was found to activate JAK1 (in terms of increasing phosphorylation at 6 h.p.i.), but had no significant effect on the rate of phosphorylation of JAK2, JAK3 and TYK2 (Fig. 1A). This induction of JAK1 activation (phosphorylation) was profoundly diminished by ouabain treatment in a dose dependent manner (Fig. 1B) and over the 6 h.p.i. of TGEV infection (Fig. 1C), as was the activation/phosphorylation of STAT1 and STAT3 (Fig. 1C). Strikingly, the JAK1 protein level was significantly decreased by ouabain treatment with or without TGEV infection (Fig. 1C). The natural cardenolides oleandrin, digoxin, digitoxin, and digoxigenin, as well as BSA-conjugated digoxin (digoxin-BSA) were also examined for their anti-TGEV effect, and all significantly downregulated JAK1 protein levels and phosphorylation levels of STAT1 and STAT3 with or without TGEV infection (Fig. 1D-a & 1D-b). Thus, cardenolides affect JAK1/STATs signalling mainly through diminishing JAK1 protein levels.  

This cardenolide-induced JAK1 diminishment/downregulation was also examined using a different coronavirus, HCoV-OC43; and host cell line, human colorectal adenocarcinoma HCT-8 cells. JAK1 protein levels were decreased by ouabain treatment in a dose dependent manner in HCT-8 cells, with or without HCoV-OC43 infection at 24 h.p.i. (Fig. 2A). Ouabain, digoxin, digitoxin, oleandrin, digitoxigenin, and digoxigenin-BSA also significantly diminished JAK1 protein levels and the phosphorylation of STAT3 in colorectal adenocarcinoma HCT-8 cells, with or without human CoV-OC43 infection (Fig. 2B-a & 2B-b). Therefore, natural cardenolides are suggested to effectively diminish the endogenous JAK1 protein levels.  

2.2. The diminishment of JAK1 is associated with the anti-TGEV activity of ouabain  

The role of JAK1 in the anti-TGEV effect of ouabain was studied in ST cells engineered to be depleted in JAK1 using a gene silencing approach. When the JAK1 depleted ST cells were infected by TGEV, TGEV replication was manifested by the decrease in nucleocapsid (N) protein level (analyzed by western analysis) (Fig. 3A-a) and significantly suppressed TGEV replication (examined by immunofluorescent assay), compared to that in control ST cells harboring shRNA (Fig. 3A-b). In addition, activation of the JAK family kinases was induced upon TGEV infection in ST within 30 min (Fig. 1C; [17]); and the JAK inhibitor CYT387 inhibited TGEV replication via diminishment of JAK2 activation in ST cells infected with TGEV [17]. Therefore, it is conceivable that CYT387 is capable of enhancing the anti-TGEV activity of ouabain, as manifested by a further decrease in TGEV N protein levels (Fig. 3B-a) and viral yields/titers (Fig. 3B-b). Therefore, the diminishment of JAK1 is associated with the anti-TGEV activity of ouabain.  

2.3. JAK1 diminishment/downregulation by ouabain was not associated with Na⁺/K⁺-ATPase  

Previously, we reported that the enhancement of PI3K_PDK1 activation by ouabain (which contributed to its anti-TGEV activity) was dependent on Na⁺/K⁺-ATPase [15]. Here, we discovered another effector, JAK1, that was diminished by ouabain, and thus also contributed to this anti-TGEV activity (Fig. 3). Since Na⁺/K⁺-ATPase is the perceptive pharmacological target of ouabain [18–20], we first dissected whether JAK1 diminishment by ouabain was associated with Na⁺/K⁺-ATPase. From RT-PCR analysis for mRNAs, only the α1 and α4 isoforms of Na⁺/K⁺-ATPase were found expressed in ST cells while α2 and α3 isoforms were detected to express in heart or brain tissues of mouse, which served as positive controls in RT-PCR for α2 and α3 isoforms (Fig. 4A). Further gene silencing of α1 or α4 isoforms did not decrease the protein level of JAK1 in ST cells (Fig. 4B & 4C-a & 4C-b). Therefore, JAK1 diminishment by ouabain is not associated with Na⁺/K⁺-ATPase.  

Other cardiac steroids such as bufalin and two innovative Na⁺/K⁺-ATPase effectors (istratepine, an inhibitor; and rostafuroxin, a modulator) [21,22] were also examined for their effect on JAK1 diminishment (Fig. 4D-a & 4D-b). Strikingly, both istaroxime and rostafuroxin, diminished JAK1 protein levels to a much lesser extent than the natural cardenolides (Figs. 1 and 2) (Fig. 4D). This result indicated that the targeting or inhibition of Na⁺/K⁺-ATPase is not necessarily associated with JAK1 diminishment, as it is for the natural cardenolides (Figs. 1 and 2) (Fig. 4D).  

Thus, we conclude that the anti-TGEV activity of ouabain caused by its diminishment of JAK1 is independent of both Na⁺/K⁺-ATPase and ouabain enhanced PI3K_PDK1 signaling axis activation.  

2.4. Protease inhibitor MG132 restored ouabain diminished JAK1 protein levels  

Cardenolides are also known to interact with estrogen, glucocorticoid, and androgen receptors [1,23,24], in addition to Na⁺/K⁺-ATPase. Accordingly, we investigated the role of these steroid receptors in ouabain-associated JAK1 diminishment. TGEV infected ST cells were treated with agonists or antagonists of glucocorticoid receptor (GR), ER, and androgen receptor (AR). We found that diindolylmethane (DIM), an AR antagonist, diminished TGEV N protein expression and significantly decreased the protein and phosphorylation levels of JAK1 (Fig. 5A). Furthermore, in TGEV infected ST cells, the protease inhibitor MG132 could rescue JAK1 protein levels reduced by ouabain, but not DIM (Fig. 5B & 5C). Moreover, the JAK1 mRNA level was not decreased by either ouabain or DIM as determined by RT-qPCR (Fig. 5D). Therefore, both ouabain and the AR antagonist DIM exert anti-TGEV activity at least in part by downregulation of JAK1, but the respective mechanisms that underlie these effects are different. Ouabain-associated JAK1 diminishment is neither dependent on Na⁺/K⁺-ATPase, nor associated with GR, ER, or AR.  

2.5. Deregulation of JAK1 by ouabain induced proteolysis was mediated by Ndfip1/2 and E3 ligase NEDD4  

A few ubiquitination proteases and phosphatases have been reported to mediate JAK1 degradation [25–27], including Ndfip1/2 by activation of the proteolysis ligase NEDD4 [25]. We thus examined these potential effectors and found that Ndfip1/2 may mediate the ouabain induced JAK1 deregulation. Herein, we knocked down the expression of either Ndfip1 or Ndfip2 (Fig. 6A) and found that depletion of either was able to significantly restore the downregulated JAK1 protein level caused by ouabain treatment (Fig. 6B). Furthermore, the NEDD4 inhibitor Heclin was also able to significantly rescue the ouabain downregulated JAK1 in a dose dependent manner (Fig. 6C).
Ndfip1/2 were reported to activate the proteolysis ligase NEDD4 and thus cause the JAK1 degradation [25]. Therefore, we conclude that Ndfip1/2 and NEDD4 are associated with ouabain induced JAK1 diminishment.

3. Materials and methods

3.1. Cells, viruses, immunofluorescent assay (IFA)

Swine testicular (ST) epithelial cells (ATCC® CRL-1746TM) and human colon adenocarcinoma cell line, HCT-8 (ATCC® CCL-244™) were obtained from American Type Culture Collection (ATCC) and passaged within six months of receipt, and further established as stock in the cell bank at early passage to ensure cell line-specific characteristics [28]. The passages 10 to 18 of ST cells and passages 8 to 15 of HCT-8 cells were used in this study. Cells and the Taiwan field isolated virulent strain of TGEV were grown and propagated; immunofluorescent assay (IFA) and phase contrast regarding TGEV infection were also described [29,30] with only two modifications: 1) test compounds were pretreated for 0.5 h; 2) ST cells were infected with TGEV at a multiplicity of infection (MOI) of 7 for all experiments, except IFA (3.5 MOI) and determination of virus titers (0.01 MOI). HCT-8 cells were cultured and maintained in Dulbecco’s Modified Eagle’s Medium (DMEM, HyClone Laboratories Inc., South Logan, USA) supplemented with 1% penicillin-streptomycin (Biological Industries Inc.), 1% non-essential Amino acids (Biological Industries Inc.), and 10% heat inactivated fetal bovine serum (FBS, Biological Industries Inc., Cromwell, CT, USA) in a humidified incubator containing 5% CO2 atmosphere at 37 °C. HCoV-OC43 (ATCC® VR1558™) were grown and propagated in HCT-8 cells cultured with DMEM and 10% FBS. For compound treatment studies, cells were then cultured in DMEM medium containing 2% FBS. Cells were then cultured with compounds for 1 h prior to HCoV-OC43 infection in MOI of 0.1. The resultant cells were harvested at 24 h.p.i. and subjected to western analyses.

3.2. Chemicals and antibodies for western blot analyses

DMSO (≥ 99.5%), digoxin (D6003, ≥ 95%, HPLC), digitoxin (D5878, ≥ 92%, HPLC), digoxigenin (D9404, 99%, TLC), ouabain (O3125, ≥ 95%, HPLC), and oleandrin (O9640, ≥ 98%, HPLC) were purchased from Sigma-Aldrich (St. Louis, MO, USA); MG132 (474790, ≥ 98%, HPLC) from Merck Millipore Calbiochem (Merck, La Jolla, CA, USA); CTX387 (S2219, ≥ 95%, HPLC) and MLN4924 (S7109, ≥ 99%, HPLC) from Selleckchem (Houston, TX, USA); Hecln (5433, ≥ 98%, HPLC) from Tocris Bioscience (Minneapolis, USA); Bufalin (15725, ≥ 98%, HPLC) from Cayman Chemical (Ann Arbor, MI, USA); Digoxin-BSA (80-ID10, HPLC) from Fitzgerald Industries (Acton, MA, USA); Rostafuroxin (T2621, ≥ 99%, HPLC) from Target Molecule Corp. (Boston, MA, USA); Istaroxime hydrochloride (HY-15718A, ≥ 99%, HPLC) from MedChem Express (Monmouth Junction, NJ, USA).

Western blotting was performed as described [30,31]. Subsequently the cell lysates were subjected to SDS-polyacrylamide gel electrophoresis for western blotting analyses with antibodies against β-actin (MAB1001, 1:1000) (Millipore Merck Chemicon, Pittsburgh, PA, USA); p-STAT1 (Y701) (catalog # 7649, 1:1000), STAT1 (catalog # 9172, 1:1000), p-STAT3 (Y705) (catalog # 9131, 1:1000), STAT3 (catalog # 9132, 1:1000), p-JAK1 (Y1022/1023) (catalog # 3331, 1:1000), JAK1 (catalog # 3344, 1:1000), p-JAK2 (Y1007/1008) (catalog # 3771, 1:1000), JAK2 (catalog # 3230, 1:1000), p-JAK3 (Y980/981) (catalog # 5031, 1:1000), JAK3 (catalog # 8863, 1:1000), TYK2 (catalog # 9312, 1:1000), p-TYK2 (Y1054/1055) (catalog # 9321, 1:1000), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (catalog # 2118, 1:2000) all from Cell Signaling Technology, MA, USA; Na+/K+ -ATPase α1 (ab9751, 1:1000) (Abcam Inc, Cambridge, UK); vinculin (GTKX109749, 1:2000) (GeneTex, CA, USA); OC43 nucleocapsid protein (N protein) (MBA9013, 1:1000) (Merck, La Jolla, CA, USA) and an antibody against TGEV N protein, as described [30]; all of these antibodies were used to detecting the respective counterpart in ST cells are described in previous reports [15,17]; and horseradish peroxidase-conjugated secondary antibodies (PerkinElmer, Inc., Waltham, MA, USA). Enhanced chemiluminescence detection reagents (Western Blot Chemiluminescence Reagent Plus; PerkinElmer, Inc., Waltham, MA, USA) were used according to the manufacturers’ instructions to detect antigen–antibody complexes. GAPDH, β-actin, or vinculin were used as internal loading controls for western analyses.

3.3. Animal study protocols

Eight-week-old female Balb/c mice (BioLASCO Taiwan Co., Ltd.) were used in this experiment. Animal study protocols for the in vivo experiments herein were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of National Health Research Institutes, Taiwan.

3.4. RNA isolation, semi-quantitative and quantitative reverse-transcriptase polymerase chain reaction (semi-RT-PCR and RT-qPCR)

The heart and brain tissue of Balb/c mice (BioLASCO Taiwan Co., Ltd.) were collected flash-frozen in liquid nitrogen. The frozen samples were homogenized using liquid nitrogen and dissolved in Trizol (Invitrogen, Waltham, MA, USA) to obtain their total RNAs, which were extracted by TRIzol Reagent (Invitrogen, Waltham, MA, USA) according to the manufacturers’ protocol, and then reverse transcribed to cDNA using SuperScript™ III (Invitrogen, Waltham, MA, USA) and oligo-dT primers. The primers used to amplify the PCR products are listed in Table 1. The amplification of target cDNA was conducted under the following conditions: 32 cycles of 94 °C for 2 min, 53 °C for 15 sec, and 72 °C for 5 min. The final PCR products were subjected to electrophoresis on a 2% agarose gel containing ethidium bromide along with DNA markers. In Fig. 4A and 6A, the amplification of respective PCR product amounts was obtained by ImageJ and normalized with the housekeeping gene GAPDH when necessary. In Fig. 5D, the changes in mRNA expression levels were determined using the ΔΔCT method with actin housekeeping genes by qPCR.

3.5. Gene silence

ST cells harbored APT1A1 shRNA (clone ID: TRCN000032624), APT1A4 shRNA (clone ID: TRCN0000432281), JAK1 shRNA #1 (clone ID: TRCN0000195125), JAK1 shRNA #2 (clone ID: TRCN0000121213), JAK2 shRNA (clone ID: TRCN0000033179), TYK2 shRNA (clone ID: TRCN0000320620), Ndfip1 shRNA #1 (clone ID: TRCN0000326242), Ndfip1 shRNA #2 (clone ID: TRCN0000195125), Ndfip1 shRNA #3 (clone ID: TRCN0000121213), and Ndfip1 shRNA #4 (clone ID: TRCN0000326242).
Fig. 2. Cardenolides diminished JAK1 protein levels and JAK1 phosphorylation, as well as the downstream phosphorylation of STAT3 in HCoV-OC43 infected HCT-8 cells. A. Ouabain decreased the protein level of JAK1 in a dose dependent manner in colorectal adenocarcinoma HCT-8 cells, with or without human CoV-OC43 infection. B. Ouabain, digoxin, digitoxin, oleandrin, digitoxigenin, and digoxin-BSA significantly diminished JAK1 protein levels and STAT3 phosphorylation in HCT-8 cells with (a) or without (b) human CoV-OC43 infection. HCT-8 cells were pretreated with cardenolides as indicated concentrations for 1 h and then infected by CoV-OC43 in MOI of 0.1. The resultant cells were harvested at 24 h.p.i. and subjected to western analyses with the antibody indicated. Two effective doses were used for each cardenolides in “B”, which were based on their effectiveness in diminishing JAK1 protein and their IC\textsubscript{50} values in TGEV inhibition [14]. *, P < 0.05; **, P < 0.01. Results shown are represented results or averages +/- SD from three independent experiments.
Fig. 3. JAK1 inhibition was associated with anti-TGEV activity. A. Depletion of JAK1 by gene silencing significantly decreased TGEV activity. A-a. TGEV N protein levels in JAK1-knockdown ST cells were examined by western analysis. A-b. TGEV infectivity, as manifested with expression of N and S (spike) proteins in JAK1-knockdown ST cells, was examined by an indirect immunofluorescent assay. The validated knocked down cells were seeded onto a 6 well-plate, 1.8 × 10⁶ cells/well, and cultured for 24 h prior to harvesting for western analysis with the antibodies indicated. **, P  <  0.01. Results shown are represented results or averages +/- SD from three independent experiments. The gene names for JAK1, JAK2, and TYK2 respectively are JAK1, JAK2, and TYK2. IFAs were shown with antibodies against S and N protein of TGEV in TGEV (3.5 MOI) infected ST cells harboring indicated shRNA at 6 h.p.i.. Phase contrast images were shown for the same field of ST cells assayed for IFA. Shown are representative of three independent experiments.

B. JAK2 inhibitor CYT387 enhanced the anti-TGEV activity of ouabain. B-a. Combined treatments of ouabain and CYT387 resulted in an additive diminishment of N protein levels by western analysis in TGEV (7 MOI) infected ST cells. B-b. Combined treatments of ouabain and CYT387 resulted in a synergistic inhibition in TGEV viral titers/replications in ST cells. ST cells were pretreated with the indicated compounds for 1 h, and then infected with TGEV in MOI of 0.01. The resultant cultured medium was harvested at 22 h.p.i and subjected to determining virus titers by an end-point assay as described in Materials and Methods. The doses of ouabain or were referred to the dose response in Fig. 1B and those of CYT387 based on previous report [17]. *, P  <  0.05; **, P  <  0.01. Results shown are averages +/- SD from three independent experiments.
Fig. 4. JAK1 diminishment by ouabain for mediating TGEV inhibition is independent of Na⁺/K⁺-ATPase. A. ST cells expressed significant levels of ATP1A1 and ATP1A4. The relative mRNA expression levels by RT-PCR of ATP1A1-A4 in ST cells or in cell lysates from mouse tissues as indicated, averages +/- SD, from three experiments were shown. Lysates from mouse tissues were used herein for PCR control. The swine specific primer pairs (shown in Table 1) for ATP1A1-A4 were used. B. Depletion of ATP1A1 by gene silencing did not result in JAK1 inhibition in in ST cells. C. Depletion of ATP1A4 by gene silencing did not result in JAK1 inhibition in ST cells. ST cells harboring sh-ATP1A1 or sh-ATP1A4 were validated and the respective gene silencing effect on the JAK1 level was examined by western analyses. Averages +/- SD, from three experiments, were shown. D. The effects of bufalin and the potent innovative Na⁺/K⁺-ATPase effectors, istaroxime and rostafuroxin on the JAK1 protein level. ST cells were pretreated with compounds for 1 h and then infected by TGEV in MOI of 7. The resultant cells were harvested at 6 h.p.i. and then subjected to western analyses with the antibody indicated. Two effective doses were used for each Na⁺/K⁺-ATPase inhibitors in D”, which were based on their effectiveness in diminishing JAK1 protein. *, P < 0.05; **, P < 0.01. Results shown are represented results or averages +/- SD from three independent experiments.

Fig. 5. Ouabain induced JAK1 degradation through ubiquitination mediated proteolysis. A. Comparison of JAK1 phosphorylation and protein levels treated by ouabain and agonists/antagonists of GR, ER and AR. B. Protease inhibitor MG132 restored ouabain diminished JAK1 protein level. C. Proteasome inhibitor MG132 did not restore the DIM diminished JAK1 protein level. D. Ouabain treatment did not decrease but increase the JAK1 transcriptional activity while DIM exhibited no significant effect as determined by RT-qPCR. ST cells were treated with ouabain, agonists/antagonists of GR, ER and AR, or MG132 as indicated at 6 h.p.i. of TGEV (MOI:7) and the resultant lysates were subjected to western analyses or RT-qPCR respectively. The effective concentration of ouabain was used based on the dose dependent experiment from Fig. 1 C. The effective concentrations of MG132 and antagonists of steroid receptor used herein were based previous report or tests done for effective doses prior their applications herein [37]. N.S., no significance; *, P < 0.05; **, P < 0.01. Averages +/- SD, from three experiments, were shown below their respective western blot.
Fig. 6. Ndfip1/2 and E3 ligase NEDD4 were associated with ouabain induced JAK1 diminishment. A. Ndfip1/2 mediated the ouabain induced JAK1 deregulation. The mRNA expression of either Ndfip1 or Ndfip2 were knockdown by gene silencing. The relative mRNA expression levels of Ndfip1 or Ndfip2 in ST cells harboring the respective Ndfip1-shRNA, Ndfip2-shRNA, or control shRNA, averages +/- SD, from three experiments were shown below their respective RT-PCR.

B. Depletion of either one of Ndfip1 or Ndfip2 restored the downregulated JAK protein level caused by ouabain treatment. Averages +/- SD, from three experiments, were shown below their respective western blot.

C. NEDD4 inhibitor Heclin restored the ouabain downregulated JAK1. ST cells were treated with Heclin or ouabain as indicated for 5 h and the resultant lysates were subjected to western analyses. The effective concentration, 250 nM, of ouabain was used based on the dose dependent experiment from Fig. 1 C. The effective concentrations of Heclin used herein were based previous report [38]. MLN4924, an NEDD8 inhibitor was used here in as a non-specific reference control and the concentrations used as reported[39]. *, P < 0.05; **, P < 0.01. Averages +/- SD, from three experiments, were shown below their respective western blot.
After validation of knockdown expression in Na+/K+-ATPase α1, Na+/K+-ATPase specific gene silencing were prepared as described previously [14]. TRC05), negative control-shRNA #2 (shLacZ, clone ID: TRCN0000231722-ID:TRCN0000365568), Ndfip2 shRNA #2 (clone ID:TRCN0000370776-ID:TRCN0000276161), Ndfip2 shRNA #1 (clone ID:TRCN0000276161), Ndfip1 shRNA #2 (shLacZ, clone ID: TRCN0000365568), Ndfip1 shRNA #1 (clone ID:TRCN0000231722-TCID = 0.69PFU (Plaque-forming unit).

The end point dilution experiment was performed as described previously [14]. Briefly, the supernatants obtained from the culture of TGEV infected ST cells (MOI of 0.01) with the treatment of indicated ouabain, CYT387, or vehicle DMSO were respectively subjected to viral titer determination via the end point dilution assay and the Reed Muench method was used to determine TCID50 (Tissue Culture Infective Dose) and measure the infectious TGEV virus titer as 10^-10^.

The statistical significance between the two groups was evaluated by the two-tailed unpaired Student’s t test; * and ** were used to denote the statistical significance for p < 0.05, and p < 0.01 respectively.

4. Discussions

Our results suggest there is another cellular target of cardenolides in addition to Na⁺/K⁺-ATPase which is associated with the proteolysis of JAK1 in TGEV infected ST cells. We investigated this mechanism and found that JAK1 deregulation contributed to the anti-coronaviral activity of natural cardenolides and that the JAK1 proteolysis upon ouabain treatment is associated with Ndfip1/2, which interacts with and activates E3 ligase NEDD4, resulting in JAK1 diminishment/inhibition of the cardenolides as antivirals.

In conclusion, an unprecedented biological function of the cardenolides independent of their Na⁺/K⁺-ATPase membrane target has been discovered. Our study has yielded novel insight into the pharmacological effects of cardenolides, which may constitute the basis for future antiviral agents and PROTACs.

Author contributions

C.W.Y. performed most of the biochemistry, and molecular biology experiments. H.Y.S., H.Y.C. and Y.Z.L. performed parts of the biochemistry, and molecular biology experiments. S.J.L. and C.W.Y. participated in the design and analysis of various experiments. S.J.L. and C.W.Y. interpreted the data and wrote the manuscript. S.J.L. supervised the experimental design, the interpretation of the data, and the composition of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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