Auranofin Suppresses Plasminogen Activator Inhibitor-2 Expression through Annexin A5 Induction in Human Prostate Cancer Cells

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Abstract
Auranofin has been developed as antirheumatic drugs, which is currently under clinical development for the treatment of chronic lymphocytic leukemia. Previous report showed that auranofin induced apoptosis by enhancement of annexin A5 expression in PC-3 cells. To understand the role of annexin A5 in auranofin-mediated apoptosis, we performed microarray data analysis to study annexin A5-controlled gene expression in annexin A5 knockdown PC-3 cells. Of differentially expressed genes, plasminogen activator inhibitor (PAI)-2 was increased by annexin A5 siRNA confirmed by qRT-PCR and western blot. Treatment with auranofin decreased PAI-2 and increased annexin A5 expression as well as promoting apoptosis. Furthermore, auranofin-induced apoptosis was recovered by annexin A5 siRNA but it was promoted by PAI-2 siRNA. Interestingly, knockdown of annexin A5 rescued PAI-2 expression suppressed by auranofin. Taken together, our study suggests that induction of annexin A5 by auranofin may enhance apoptosis through suppression of PAI-2 expression in PC-3 cells.

Key Words: Annexin A5, Apoptosis, Auranofin, Plasminogen activator inhibitor-2

INTRODUCTION
Auranofin is a gold compound that has been used for rheumatoid arthritis (Kean et al., 1997). As auranofin is able to prevent various crucial inflammatory pathways, it is considered as a new candidate for anticancer agents (Han et al., 2008). For example, auranofin reduces NF-κB activation via IκB kinase suppression and it decreases generation of tumor necrosis factor (TNF)-α in murine macrophage (Jeon et al., 2000). Auranofin induces apoptosis through the inhibition of IL-6-induced JAK/STAT and NF-κB pathway in myeloma cells (Stern et al., 2005; Kim et al., 2007; Nakaya et al., 2011). In addition, auranofin promotes apoptosis via FOXO3 activation or inhibition of thioredoxin reductase in human ovarian cancer cells (Marzano et al., 2007; Park et al., 2014). Phosphoinositide 3-kinase (PI3K)/Akt pathway is also inhibited by auranofin in non-small cell lung cancer cells (Li et al., 2016). Recently, Phase I/II clinical trials of auranofin to treat chronic lymphocytic leukemia has been completed (Liu et al., 2014; Clinicaltrials.gov, 2016). Moreover, our recent study suggested that auranofin leads to annexin A5 expression and translocation into mitochondria causing mitochondrial apoptosis through VDAC oligomerization in human prostate cancer cells (Park and Chun, 2014).

Annexin A5 is the annexin family protein that binds to phospholipids in a calcium-dependent manner. It was discovered from human placenta for the first time at the end of 1970s (Bohn and Kraus, 1979; Boersma et al., 2005). Annexin A5 is identified in blood vessel where it acts as a blood anticoagulation factor and it builds voltage-dependent calcium channel in phospholipid bilayers (Reutelingsperger et al., 1985; De-mange et al., 1994). The property of annexin A5 that selectively binds to negative charged phospholipid phosphatidylserine (PS) has been used to distinguish the apoptotic cells from viable cells using flow cytometry analysis for many years (Koopman et al., 1994; Vermes et al., 1995). Annexin A5 induces rat Leydig cell proliferation through RhoA/Rho-associated protein kinase pathway via Ect2 protein (Jing et al., 2015). Furthermore, annexin A5 suppresses protein kinase activity by directly interacting with protein kinase C (PKC) and annexin A5-like peptide triggers apoptosis by binding to cytoplasmic domain of integrin α5β1S (Rothhut et al., 1995; Cardó-Vila et al., 2003).
However, the role of annexin A5 itself has not yet been clarified. Recently, annexin A5 has been reported as a new mediator of cisplatin-induced apoptosis by inducing voltage-dependent anion channel (VDAC) oligomerization in human kidney epithelial cells (Kwon et al., 2013; Jeong et al., 2014).

Plasminogen activator inhibitor (PAI)-2 is a member of serpin protein family that inhibits serine protease in the blood coagulation cascade in human (Kruithof et al., 1995). PAI-2 binds and inhibits urokinase-type plasminogen activator (uPA) which activates precursor plasminogen to plasmin (Irving et al., 2000). This inhibition of PAI-2 decreases extracellular matrix (ECM) degradation by regulating uPA/urokinase-type plasminogen activator receptor (uPAR) signaling (Croucher et al., 2008). Although PAI-2 is known as an inhibitor of uPA, PAI-2 is considered as an antiapoptotic protein and strong prognostic marker in various cancer cells. First evidence of PAI-2 as an antiapoptotic factor was came from linkage to Bcl-2 because of their proximity in genome and similar structure (Silverman et al., 1991; Medcalf and Stasinopoulos, 2005). Some studies show that TNF-α-induced apoptosis can be suppressed by PAI-2 expression in several cancer cells and overexpression of PAI-2 inhibits apoptosis (Kumar and Baglioni, 1991; Dickinson et al., 1995; Zhou et al., 2001). Moreover, high expression of PAI-2 in endometrial and colorectal cancer is also shown to be closely correlated with poor prognosis (Ganesh et al., 1994; Nordeneng et al., 2002).

Because annexin A5 may play an important role in auranofin-mediated apoptosis and PAI-2 may have antiapoptotic roles, we wanted to determine whether annexin A5 is able to regulate PAI-2 expression. In this study, we explored how annexin A5 controls PAI-2 expression in auranofin-mediated apoptosis in PC-3 cells and we found that auranofin downregulates PAI-2 level via the induction of annexin A5 expression to augment apoptotic pathway.

MATERIALS AND METHODS

Reagents

Auranofin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) and RPMI 1640 medium were from HyClone (Logan, UT, USA). Neon transfection system and BCA protein assay kit were from Thermo Scientific (Rockford, IL, USA). Enhanced chemiluminescence (ECL) kit was from Bionote (Gyeonggi, Korea). Moloney murine leukemia virus (M-MLV) reverse transcriptase and RNase inhibitor (RNasin) were purchased from Promega (Madison, WI, USA). Total RNA was extracted using Ribospin™ Total RNA kit (GeneAll, Seoul, Korea). Whole RNA (500 ng) was reverse transcribed at 37°C for 1 h for 20 µl volume containing 5x RT buffer, 10 mM dNTPs, 40 units of RNase inhibitor, 200 units of M-MLV reverse transcriptase, and 100 pmole of oligo-dT primer. Subsequently, 0.8 µl of the reaction mixture from each sample was amplified with 10 pmole of each oligonucleotide primer, 0.2 mM dNTPs, 1.5 mM MgCl₂, and 1.25 units of Taq DNA polymerase in a final volume of 25 µl. PCR was performed as follows: one cycle of 95°C for 30 sec, followed by 40 cycles of denaturation at 95°C for 5 sec, annealing according to each primer’s melting temperature (Tm) for 30 sec, and extension at 72°C for 15 sec. Human PAI-2 cDNA was amplified using a sense primer (5'-AACCTAGAGAGAGTAGATTC-3') and an antisense primer (5'-CAAGCATTCCAGGACCGT-3'). Human 18S rRNA cDNA was amplified using a sense pri-

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cDNA was hybridized to the GeneChip using a biotinylated dideoxynucleotide. Fragmented end-labeled and end-labeled by terminal transferase reaction incorporation using a dNTP mix containing dUTP. The cDNA was then regenerated through a random-primed reverse transcription reaction and purified with the Affymetrix sample cleanup module. cDNA template through an IVT (in vitro transcription) reaction and RNA (cRNA) was generated from the double-stranded cDNA using a random hexamer incorporating a T7 promoter, amplified from each sample was converted to double-strand cDNA using a Rotor-Gene Q (Qiagen) with a SYBR green I PCR assay reagents. Extracted proteins (20 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels, and were electrophoretically transferred onto polyvinylidene difluoride (PVDF) membrane. Membranes were blocked in Tris-buffered saline containing 5% (w/v) nonfat dried milk for 3 h at 4°C and then were incubated for overnight with primary antibody at a 1:1000 dilution in Tris-buffered saline. After incubating with HRP-conjugated secondary antibody for 2 h at 4°C, proteins were visualized by an ECL and the band intensity was analyzed by ChemiDoc XRS densitometer and quantified by Quantity One software (Bio-Rad, Richmond, CA, USA).

Western blot analysis

Cells were solubilized with lysis buffer (pH 7.4) containing 50 mM Tris.Cl, 150 mM NaCl and 1% Nonidet P-40. Protein concentration was measured using BCA protein assay reagents. Extracted proteins (20 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels, and were electrophoretically transferred onto polyvinylidene difluoride (PVDF) membrane. Membranes were blocked in Tris-buffered saline containing 5% (w/v) nonfat dried milk for 3 h at 4°C and then were incubated for overnight with primary antibody at a 1:1000 dilution in Tris-buffered saline. After incubating with HRP-conjugated secondary antibody for 2 h at 4°C, proteins were visualized by an ECL and the band intensity was analyzed by ChemiDoc XRS densitometer and quantified by Quantity One software (Bio-Rad, Richmond, CA, USA).

Microarray data analysis

Affymetrix (Affymetrix, Santa Clara, USA) GeneChip® Human Gene 2.0 ST oligonucleotide arrays were used for global gene expression analysis. The sample preparation was performed according to the instructions and recommendations provided by the manufacturer. Total RNA was isolated using Ribospin™ Total RNA kit. RNA quality was assessed by Agilent 2100 bioanalyzer using the RNA 6000 Nano Chip (Agilent Technologies, Amstelveen, The Netherlands), and quantity was determined by ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., DE, USA). Briefly, 300 ng of total RNA from each sample was converted to double-strand cDNA using a random hexamer incorporating a T7 promoter, amplified RNA (cRNA) was generated from the double-stranded cDNA template through an IVT (in vitro transcription) reaction and purified with the Affymetrix sample cleanup module. cDNA was regenerated through a random-primed reverse transcription using a dNTP mix containing dUTP. The cDNA was then fragmented by UDG and APE 1 restriction endonucleases and end-labeled by terminal transferase reaction incorporating a biotinylated dideoxynucleotide. Fragmented end-labeled cDNA was hybridized to the GeneChip® Human Gene 2.0 ST arrays for 17 h at 45°C and 60 rpm. After hybridization, the chips were stained and washed in a Genechip Fluidics Station 450 (Affymetrix) and scanned by using a Genechip Array scanner 3000 7G (Affymetrix). Expression data were normalized and log2 transformed using the robust multichip average (RMA) method implemented in the Bioconductor package RMA (Bolstad et. al, 2003; Irizarry et al, 2003). To reduce noise for the significance analysis, probe sets that did not show detection call rate at least 50% of the samples in the comparison were filtered out. Highly expressed genes that showed a 2-fold change in expression were selected. The results were classified using hierarchical clustering algorithms implemented in TMEV software 4.0 (Eisen et al., 1998).

Statistical analysis

Statistical analysis was implemented by using one-way analysis of variance, followed by Dunnett’s pairwise multiple comparison t-test with GraphPad Prism software (GraphPad Software Inc., San Diego, USA) when appropriate. The difference was considered statistically significant at *p<0.05.

RESULTS

Depletion of PAI-2 expression promotes apoptosis

To confirm the function of PAI-2 as an antiapoptotic factor in PC-3 cells, PAI-2 mRNA level was knocked down in PC-3 cells using siRNA (37.5 nM) for 48 h. Cells treated with PAI-2 siRNA showed decreased cell viability about 20% relative to control group (Fig. 1A). Moreover, we examined the protein levels of caspase 3, PARP, or Bcl-2 in PAI-2 knockdown cells. Cleaved caspase 3 and PARP levels were increased and Bcl-2 level was decreased after transfection of PAI-2 siRNA (Fig. 1B). These results indicated that PAI-2 is able to inhibit apop-
An Annexin A5 suppresses PAI-2 expression

To identify whether annexin A5 regulates specific gene expression in PC-3 cells, cells were transfected with annexin A5 siRNA (37.5 nM) for 48 h. Annexin A5-depleted cells were analyzed with Affymetrix GeneChip® Human Gene 2.0 ST Array. Of approximately 30,000 differentially expressed genes in microarray data, 33 genes were increased when annexin A5 was knocked down in PC-3 cells (Table 1). Because PAI-2 gene expression was significantly increased (2.6-fold) in knockdown cells and PAI-2 is considered as an antiapoptotic factor in cancer cells, we chose this gene to see whether annexin A5 modulates PAI-2 gene expression to control apoptosis in PC-3 cells. To confirm that annexin A5 expression affects PAI-2 level, annexin A5 siRNA (37.5 nM) or annexin A5 overexpression plasmid (5 μg) was used. As results, PAI-2 mRNA level was increased 1.8-fold in knockdown cells of annexin A5 and decreased about 20% when cells were overexpressed with annexin A5 (Fig. 2A). As shown in Fig. 2B, protein level of PAI-2 was also upregulated about 1.4-fold in annexin A5 siRNA-treated cells and downregulated about 10% in annexin A5 overexpressing cells. These results indicate that annexin A5 may inhibit PAI-2 mRNA and protein expression in PC-3 cells.

Auranofin inhibits PAI-2 expression level via annexin A5 induction

To examine that auranofin inhibits cell proliferation and drives apoptosis in PC-3 cells, various concentrations (0, 0.25, 0.5, or 1 μM) of auranofin were treated and cell viability and apoptosis assay was performed (Fig. 3). When cells were treated with auranofin, cell viability was decreased about 20% at 0.5 μM and 75% at 1 μM (Fig. 3A). To confirm whether auranofin is able to promote apoptosis in PC-3 cells, flow cytometry analysis was performed. The percentage of total apoptotic cell was about 30% at 0.5 μM and 75% at 1 μM (Fig. 3B). These data showed that auranofin induces apoptosis in PC-3 cells in a concentration-dependent manner. To identify whether annexin A5 and PAI-2 are expressed in an opposite way in auranofin-induced apoptosis, the miRNA and protein

### Table 1. Microarray data analysis in annexin A5 knockdown PC-3 cells

| Gene symbol (representative) | Log2 ratio | Absolute fold change | Gene description |
|-----------------------------|------------|----------------------|------------------|
| HSD3B2                      | 1.62       | 3.07                 | Hydroxy-Δ5-steroid dehydrogenase, 3β- and steroid Δ-isomerase 2 |
| PAI-2                       | 1.41       | 2.66                 | Plasminogen activator inhibitor-2 |
| KRTAP3-1                    | 1.34       | 2.53                 | Keratin associated protein 3-1 |
| VTRNA1-1                    | 1.33       | 2.52                 | Vault RNA 1-1 |
| LOC66813                    | 1.32       | 2.49                 | DEAH (Asp-Glu-Ala-His) box helicase 9 pseudogene |
| CCL3L3                      | 1.27       | 2.41                 | Chemokine (C-C motif) ligand 3-like 3 |
| OR52I1                      | 1.26       | 2.39                 | Olfactory receptor, family 52, subfamily I, member 1 |
| TBC1D4-AS1                  | 1.24       | 2.36                 | TBC1D4 antisense RNA 1 |
| TAS2R20                     | 1.22       | 2.33                 | Taste receptor, type 2, member 20 |
| TRNA6                       | 1.21       | 2.32                 | Transfer RNA isoleucine 6 (anticodon UAU) |
| SNRPN                       | 1.21       | 2.31                 | Small nuclear ribonucleoprotein polypeptide N |
| AMY2A                       | 1.21       | 2.31                 | Amylase, α 2A (pancreatic) |
| CLYBL                       | 1.19       | 2.29                 | Citrate lyase β-like |
| CRYZL1                      | 1.18       | 2.27                 | Crystallin, ζ (quinone reductase)-like 1 |
| FAM74A2                     | 1.17       | 2.25                 | Family with sequence similarity 74, member A2 |
| OR52T2                      | 1.16       | 2.23                 | Olfactory receptor, family 5, subfamily T, member 2 |
| YME1L1                      | 1.15       | 2.22                 | YME1-like 1 ATPase |
| SNORD114-4                  | 1.14       | 2.20                 | Small nucleolar RNA, C/D box 114-4 |
| SNORD20                     | 1.13       | 2.19                 | Small nucleolar RNA, C/D box 20 |
| RNASP38                     | 1.12       | 2.18                 | RNA, SS ribosomal pseudogene 38 |
| MYH2                        | 1.07       | 2.11                 | Myosin, heavy chain 2, skeletal muscle, adult |
| PRAMEF3                     | 1.07       | 2.10                 | PRAME family member 3 |
| KRTAP13-4                   | 1.07       | 2.09                 | Keratin associated protein 13-4 |
| TRIM43B                     | 1.06       | 2.09                 | Tripartite motif containing 43B |
| IL13RA2                     | 1.05       | 2.07                 | Interleukin 13 receptor α2 |
| MS4A4E                      | 1.03       | 2.04                 | Membrane-spanning 4-domains, subfamily A, member 4E |
| IFI44                       | 1.02       | 2.03                 | Interferon-induced protein 44 |
| RNU7-60P                    | 1.01       | 2.02                 | RNA, U7 small nuclear 60 pseudogene |
| PCDH11X                     | 1.01       | 2.01                 | Protocadherin 11 X-linked |
| OR52M1                      | 1.00       | 2.00                 | Olfactory receptor, family 52, subfamily M, member 1 |
| F3                          | 1.00       | 2.00                 | Coagulation factor III (thromboplastin, tissue factor) |
| HIST1H3J                    | 1.00       | 2.00                 | Histone cluster 1, H3j |
| RNASP409                    | 1.00       | 2.00                 | RNA, SS ribosomal pseudogene 409 |

Cells were transfected with annexin A5 siRNA for 48 h. Gene expressions were analyzed with Affymetrix GeneChip® Human Gene 2.0 ST Array. Cut off ≥ 2-fold (p<0.05).
levels of annexin A5 and PAI-2 were measured by qRT-PCR and western blot analysis, respectively. Treatment with auranofin of PC-3 cells for 24 h led to increase of annexin A5 mRNA and protein expression and decrease of PAI-2 mRNA and protein expression in concentration-dependent manners (Fig. 4). These findings suggest that annexin A5 and PAI-2 may play an antagonistic role each other in auranofin-induced apoptosis in PC-3 cells.

Relationship between annexin A5 and PAI-2 in auranofin-induced apoptosis

To examine the role of annexin A5 in auranofin-treated PC-3 cells, annexin A5 siRNA (37.5 nM) was transfected with or without auranofin (1 μM) into cells. As shown in Fig. 5A, treatment with auranofin in the presence of annexin A5 siRNA recovered cell viability about 20% compared to scrambled siRNA-treated cells. Apoptosis assay using flow cytometry also revealed that auranofin-induced apoptosis was prevented by annexin A5 siRNA about 15% (Fig. 5B). These results demonstrate that annexin A5 may play an important role in auranofin-induced apoptosis. To further determine the function of PAI-2, cells were treated with auranofin (1 μM) for 24 h in the presence of PAI-2 siRNA (37.5 nM). As shown in Fig. 6A, treatment with PAI-2 siRNA resulted in decreasing cell viability about 10% compared to the control cells. Flow cytometry analysis also showed that increased apoptosis by auranofin was enhanced by knockdown of PAI-2 about 10% (Fig. 6B). These data show that PAI-2 may act as an antiapoptotic factor in auranofin-induced apoptosis. In an effort to investigate relationship between annexin A5 and PAI-2 in auranofin-mediated apoptosis, cells transfected with annexin A5 siRNA were treated with auranofin (1 μM) for 24 h to induce apoptosis. After treatment with annexin A5 siRNA and 1 μM of auranofin, PAI-2 mRNA and protein levels were determined. As shown in
Auranofin induces apoptosis in PC-3 cells. (A) Cell viability assay. PC-3 cells were treated with auranofin at various concentrations (0, 0.25, 0.5, or 1 μM) for 24 h. After incubation, the absorbance was measured at 450 nm. The percentage of cells surviving in each group relative to the control was calculated. The data are showed as a mean ± SD (n=3). *Significantly different from control (p<0.05). (B) Apoptosis assay using flow cytometry. PC-3 cells were treated with auranofin at various concentrations (0, 0.25, 0.5, or 1 μM) for 24 h. The cells were stained with Muse™ annexin V dead cell kit. After incubation for 20 min at room temperature, Muse™ cell analyzer assessed the percentage of apoptotic cells.

Fig. 3. Auranofin induces apoptosis in PC-3 cells. (A) Cell viability assay. PC-3 cells were treated with auranofin at various concentrations (0, 0.25, 0.5, or 1 μM) for 24 h. After incubation, the absorbance was measured at 450 nm. The percentage of cells surviving in each group relative to the control was calculated. The data are showed as a mean ± SD (n=3). *Significantly different from control (p<0.05). (B) Apoptosis assay using flow cytometry. PC-3 cells were treated with auranofin at various concentrations (0, 0.25, 0.5, or 1 μM) for 24 h. The cells were stained with Muse™ annexin V dead cell kit. After incubation for 20 min at room temperature, Muse™ cell analyzer assessed the percentage of apoptotic cells.

Fig. 4. Auranofin suppresses PAI-2 expression through annexin A5 induction in PC-3 cells. (A) qRT-PCR. PC-3 cells were treated with auranofin at various concentrations (0, 0.25, 0.5, or 1 μM) for 24 h. After harvesting, total RNA was isolated and qRT-PCR was performed to determine annexin A5 and PAI-2 mRNA expression levels. 18S rRNA was used as a RNA control. The data are showed as a mean ± SD (n=3). *Significantly different from control (p<0.05). (B) Western blot analysis. PC-3 cells were treated with auranofin at various concentrations (0, 0.25, 0.5, or 1 μM) for 24 h. Cells were harvested and total cellular proteins were extracted. Extracted proteins were separated by SDS-PAGE (10%) and western blot analysis was performed with specific antibodies. β-actin level was determined as a loading control. Quantity one software were used to measure the intensities of protein bands. Relative protein expression level was determined by the ratio of target protein to β-actin. The data are showed as a mean ± SD (n=3). *Significantly different from control (p<0.05).

Fig. 7A, PAI-2 mRNA level was increased about 1.8-fold compared to the control level. Auranofin suppressed PAI-2 mRNA expression about 40% but annexin A5 siRNA significantly recovered auranofin-mediated PAI-2 mRNA suppression about 2-fold. Western blot data also showed that annexin A5 siRNA prevents auranofin-mediated PAI-2 downregulation (Fig. 7B). Taken together, these results suggest that induction of annexin A5 by auranofin may suppress PAI-2 expression.

DISCUSSION

PAI-2 is previously known to protect TNF-α-induced apopto-
in annexin A5 knockdown cells and was decreased in annexin A5-overexpressing cells. Previous study revealed that auranofin increases annexin A5 expression and translocation into mitochondria to induce apoptosis through oligomerization of VDAC in PC-3 cells (Park and Chun, 2014). Hence, we treated auranofin to induce annexin A5-mediated apoptosis and observed the PAI-2 level resulting in reduction of PAI-2 expression in a concentration-dependent manner. Interestingly, when annexin A5 siRNA was treated into cells, auranofin-induced apoptosis was significantly recovered. However, PAI-2 siRNA promoted apoptosis by auranofin. Furthermore, annexin A5 siRNA restored auranofin-mediated PAI-2 suppression. Thus, our results suggested that induction of annexin A5 by auranofin may augment apoptosis through suppression of PAI-2 expression.

Previous reports suggested that annexin A5 may suppress PKC activity in mesangial cells (Rothhut et al., 1995). Auranofin also inhibits the catalytic activity of PKC (Mahoney et al., 1989). Phorbol 12-myristate 13-acetate (PMA), which is the activator of PKC, induces PAI-2 expression by controlling
and western blot analysis was performed with specific antibodies. Extracted proteins were separated by SDS-PAGE (10%) with annexin A5 siRNA for 24 h and then treated with auranofin (1 μM) for 24 h. After treatment, total RNA was isolated and qRT-PCR was performed to determine annexin A5 and PAI-2 mRNA expression levels. 18S rRNA was used as a RNA control. Therefore, there is a possibility that annexin A5 induction during auranofin-mediated apoptosis inhibits PKC pathway resulting in PAI-2 suppression. To clarify this mechanism, further studies will be required.

In summary, our results reveal that increased annexin A5 during auranofin-mediated apoptosis prevents PAI-2 expression in PC-3 cells, and this mechanism could be a novel pathway to understand annexin A5-mediated apoptosis as well as mitochondria-dependent annexin A5 pathway. Lastly, we suggest that annexin A5 could be considered as an apoptotic factor and targeted for new anticancer therapy.

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