Cytoprotective Role of *Phyllanthus urinaria* L. and Glutathione-S Transferase Pi in Doxorubicin-induced Toxicity in H9c2 Cells

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Objective: *To examine cytoprotective effect of phyllanthus urinaria (PU) ethanolic extract in doxorubicin (DOX)-induced toxicity. The research focus was on the mechanism of action in association with the expression and localization of glutathione-S transferase (GST) in cardiac H9c2 cells.*
Material and Method: *The presence of GST isoforms was evaluated in H9c2 cells using western blot analysis and confocal immunofluorescence visualization. Cells were then treated with DOX in the presence and absence of PU and several cytoprotective indices were evaluated, including the expression of the rate-limiting enzyme for glutathione synthesis, γ-glutamylcysteine synthetase (γ-GCS), manganese superoxide dismutase (MnSOD), copper-zinc SOD (CuZnSOD), and GST activity from cell lysate. The investigations for GST-mediated cytoprotection from DOX-induced oxidative damage were further carried out by SiRNA transfection and apoptosis detection using TUNEL assay.*
Results: *GST Pi (GSTP) was predominantly expressed in H9c2 cells compared with GST Alpha and GST Mu. Treatment with PU protected against the cardiotoxicity of DOX by influencing the nuclear localization of GSTP without significantly affecting the enzymatic activity. Suppression of GSTP expression by RNA interference potentiated the accumulation of DOX in the nucleus and enhanced apoptosis as evaluated by TUNEL assay. Treatment with PU had a cytoprotective effect by reducing cellular levels of DOX with enhanced nuclear localization of GSTP in myocardiac cells.*
Conclusion: *The cytoprotective mechanism of PU against DOX cardiotoxicity partially involved the presence of GSTP. Thus, PU extracts may be used as an alternative source of antioxidants with distinctive mechanisms of action that may be suitable for specific types of oxidative insults.*

Keywords: *Apoptosis, Doxorubicin, Glutathione S-transferase pi, Myoblasts, cardiac, Oxidative stress, Phyllanthus*

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Doxorubicin (DOX) is an anticancer drug that exerts its cytotoxicity primarily by interfering with topoisomerase II and binding to DNA to generate DNA cleavable complexes[1,2]. The generation of reactive oxygen species (ROS) is also implicated in the cellular damage and apoptosis caused by DOX[3,4]. Cardiac tissue is one of the major targets of DOX and often leads to compromised cancer therapy[5]. Although several pharmaceutical modifications have been made to alleviate the cardiotoxicity of DOX (*e.g.*, non- and pegylated liposomal encapsulations) the risk of

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cardiac events still remains when high doses of the drug are administered[6]. A current alternative strategy to lessen the cardiotoxicity of DOX is the utilization of antioxidants from a wide range of molecular structures and functions including vitamins, enzymes, iron chelators, and complex molecules from plant extracts[7-9].

We have previously reported that ethanolic extract from *phyllanthus urinaria* L. (PU) protected against the toxicity of DOX in a cardiac myoblast cell line H9c2[10]. The ethanolic extract provided both exogenous and endogenous antioxidative defenses but the precise mechanisms involved have not been defined. Several studies have shown that the phase II metabolizing enzyme glutathione-S-transferase (GST, EC 2.5.1.18) plays a significant role in the detoxification of DOX[11,12]. To date, seven classes of cytosolic GST have been identified in mammalian species; Alpha (GSTA), Mu (GSTM), Pi (GSTP), Sigma (GSTS), Theta (GSTT), Omega (GSTO), and Zeta (GSTZ), along with one class of mitochondrial GST (Kappa, GSTK)[13]. However, the distribution of these classes of enzymes can be altered under certain circumstances and is dependent on gender or cell type[14].

GST classes that are selectively expressed in mouse tissues are GSTA, GSTM and GSTP[14,15]. Therefore, these three classes of GST have been the most extensively studied among other GST classes with regard to cytoprotection against oxidative stress. In addition, GSTP is particularly related to enzyme-mediated chemoprevention and anticancer drug resistance[16-19]. Recent studies have demonstrated that over-expression of GSTA protected against DOX-induced cell death in H9c2 cells[20] and small cell lung cancer H69 cells[21]. However, the expression and protective function of endogenous GST classes have not been reported in this cell type[22]. Thus, in this study, we investigated the protective effect of PU against the cardiotoxicity of DOX with regard to the expression, function and intracellular localization of GST. In addition, the expression of other antioxidative enzymes such as superoxide dismutase (SOD) and γ-glutamylcysteine synthetase (γ-GCS, the rate-limiting enzyme in glutathione synthesis) were examined.

Material and Method

Materials

Antibodies against GSTM1-1, GSTA1-1, and GADPH were procured from Calbiochem (San Diego, CA). GSTP was purified from human placenta, and a polyclonal antibody against human GSTP was obtained by immunizing rabbits as described previously[23]. Peroxidase-conjugated secondary antibodies against rabbit IgG and mouse IgG were obtained from Dako (Denmark). The other chemicals and reagents used in the study were of high quality grade from Sigma Aldrich (St. Louis, MO) or Wako Pure Chemicals (Osaka, Japan). The ethanolic extract of *Phyllanthus urinaria* (PU) was obtained from Medicinal Plant Research Institute, Department of Medical Science, Ministry of Public Health, Thailand as described previously[10].

Cell culture and treatment protocol

H9c2 cells, a clonal line derived from embryonic rat heart, were obtained from American Type Culture Collection (ATCC, CRL-1446). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere of 95% air and 5% CO2 at 37°C. Low serum medium (1% FBS) was added to the culture 24 h prior to any treatment performed. PU pretreatment was conducted 1 h prior to the addition of DOX and throughout the incubation period as indicated in the figures. THP-1 cells (human acute monocytic leukemia cell line; ATCC, TIB-202) were maintained in RPMI medium supplemented with 10% FBS.

Cell viability

Cells were treated with DOX (1 μM) for 24 h with or without PU (10 μg/ml) pretreatment. Cell survival was evaluated using crystal violet staining as previously described [10]. Briefly, cells were washed with phosphate-buffered saline (PBS, pH 7.2), fixed with 10% buffered formalin, and then stained with a 0.1% crystal violet solution. Cell survival was quantified by dissolving the crystal violet-stained nuclei in 50 mM sodium citrate solution in water/EtOH (1:1) and measuring the absorbance at 595 nm.

Immunoblot analysis

Cells were harvested and lysed in lysis buffer [20 mM Tris-HCl (pH 7.2), 130 mM NaCl, and 1% NP-40 including protease inhibitors (20 μM PMSF, 50 μM pepstatin, and 50 μM leupeptin)]. Cell lysates were normalized for protein content using a BCA protein assay kit (Pierce, Rockford, IL). Protein samples were separated by 7.5 or 10% SDS-PAGE under reducing conditions and then transferred to a nitrocellulose membrane as described previously[24]. The membrane was blocked with 5% skim milk in TBS [10 mM Tris-HCl (pH 7.5) and 150 mM NaCl] and then incubated at 4°C

overnight with the primary antibody in TBS containing 0.1% Tween 20. The blots were washed and then incubated with the peroxidase-conjugated secondary antibodies for 1 h at room temperature. Following several washes, the membrane was developed using the ECL chemiluminescence detection kit (Amersham Biosciences) according to the manufacturer's instructions.

Confocal fluorescence microscopy

H9c2 cells (50,000 cells) were grown on a glass cover slip for 24 h. After being treated with DOX or PU at various time points, cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 1% Triton X-100 in PBS for 10 min. Following the blocking step (1% BSA in PBS), the slides were incubated with primary antibody for 1 h at room temperature, and washed with PBS. The immunoreactivity of primary antibodies was detected with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit immunoglobulins (Cappel, Durham, NC). Hoechst 33342 was used for nuclear staining. After three washes, the stained cells were mounted in Vectashield medium, visualized under a Carl Zeiss LSM5 microscope (Carl Zeiss, Jena, Germany), and analyzed using PASCAL analytic software.

GST activity

Total GST activity was determined by measuring the rate of conjugation of 1-chloro-2, 4-dinitrobenzene (CDNB) with reduced glutathione as previously described[25]. Briefly, GST activity was measured in the presence of 1 mM GSH and 1mM CDNB in 0.1 M sodium phosphate buffer (pH 6.5). The GS-DNB conjugate was detected at 340 nm and the rate of increase in absorbance is directly proportional to the GST activity in the sample.

Flow cytometry for intracellular DOX measurement

H9c2 cells (1×10^6) were incubated with 1 μM DOX for the periods indicated in the figures with or without pretreatment with PU (1 mg/ml). Cells were harvested using trypsin and washed with PBS. The intracellular accumulation of DOX was subsequently measured by flow cytometry in the FL3 emission spectrum (for detecting red fluorescence) on a Beckman Coulter Cytomics FC500 (Becton-Dickinson, San Jose, CA) and analyzed with Beckman CXP software.

TUNEL assay

The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was performed using an ApopTag Plus Fluorescein in situ Apoptosis Detection Kit (Chemicon International). Briefly, approximately 1 to 2×10^6 cells were harvested, fixed in 70% ethanol, and treated with terminal deoxynucleotidyl transferase for 1 h followed by fluorescein isothiocyanate (FITC) conjugated anti-digoxigenin for 1 h at room temperature. Cells were washed twice with 0.1% Triton X-100/PBS, and re-suspended in propidium iodide containing RNase A. Fluorescence intensity was detected in the FL1 (530/30 filter) emission spectrum using Becton Dickinson FACScan and analyzed with Beckman CXP software.

RNA interference using SiRNA

SiRNA for GSTP was generated by Qiagen based on GenBank database accession numbers NM012577 and NM138974. The sequence was 5'-UAUGGUAACCACCUCCUCC-3'. Transfection was performed using Lipofectamine 2000 (Invitrogen) in OPTI-MEM medium in 6-well plates according to the manufacturer's instructions. Briefly, 3 μl of Lipfectamine 2000 and 1.5 μl of SiRNA (stock 20 μM) were each diluted in 250 μl of OPTI-MEM medium and allowed to sit at room temperature for 5 min. Then, each mixture was combined and further incubated at room temperature for 20 min. The mixture was overlaid in cell cultures and incubated for 6 h at 37°C. The cultures were then given fresh medium and the silencing effect was examined after 48 h.

Statistical analysis

Data are expressed as the mean $\pm$ SEM for at least three independent experiments. Statistical analysis was performed using Student's t-test or one-way ANOVA where appropriated. A value of $p < 0.05$ was deemed significant.

Results and Discussion

GSTP subtype is predominant in H9c2 cells

We have initially investigated the GST classes in H9c2 cells using immunoblot analysis and fluorescence microscopy. It appeared that GSTP is the most abundant while two other classes GSTA and GSTM were not detected. Rat liver lysate and THP-1 cells were used as positive controls (Fig. 1A). The GSTP in H9c2 cells was concentrated in the nucleus but also could be detected in the cytoplasm (Fig. 1B). Thus, the following experiments focused on the study of GSTP.

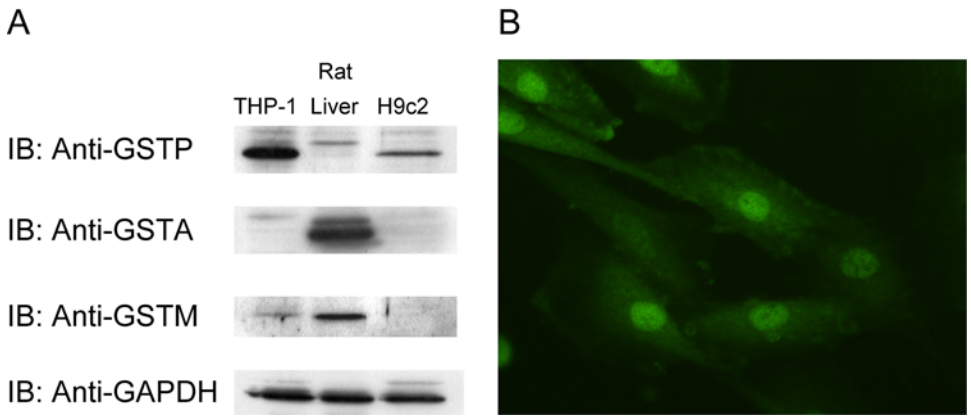

Fig. 1 Expression of GST isozymes in H9c2. (A) Immunoblots (IB) show the expression of GST subtypes. (B) The intracellular distribution of GSTP was determined using anti-GSTP antibody under a fluorescence microscope as described in Material and Method

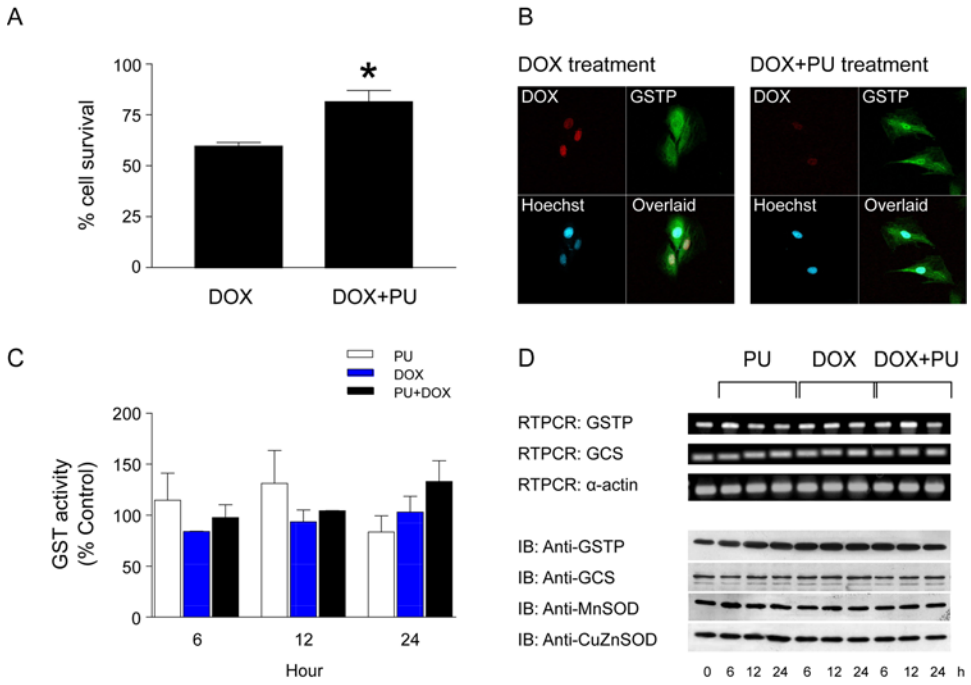

Fig. 2 Cytoprotective effects of PU, GSTP, and other antioxidative enzymes (A) PU (10 μg/ml) significantly increased H9c2 cell survival after treated with DOX (1 μM) for 24 h ($p < 0.05$). (B) Representative fluoromicroscopic pictures of cells treated with DOX (1 μM) for 24 hr alone or cells pretreated with PU (10 μg/ml) (DOX + PU). (C) Total GST activity of whole cell lysates of H9c2 treated with vehicle (CTRL), PU (10 μg/ml), DOX (1 μM), or PU (10 μg/ml) plus DOX (1 μM) for 6, 12 and 24 h. Data are presented as mean ± SD for three separate experiments, (D) Expression levels of MnSOD, CuZnSOD, GSTP, and GCS were evaluated by RT-PCR and immunoblotting (IB). The RT-PCR products from equal amounts of total RNA extracted (0.5 μg) from treated cells were separated by 1% agarose gel and visualized by ethidium bromide staining. Antibodies for GSTP, GCS, MnSOD, and CuZnSOD were used for immunoblotting as described in Material and Method

PU decreased DOX accumulation in the nucleus through the function of GSTP

PU significantly protected against the toxicity of DOX by increasing cell survival at 24 h following incubation with 1 mM DOX (Fig. 2A). The intracellular accumulation of DOX and expression of GSTP in H9c2 cells was observed by fluorescence microscopy. In DOX-treated cells, DOX was highly accumulated in the nucleus (red fluorescence), and GSTP disappeared from the nucleus (green fluorescence) (Fig. 2B Upper panel). In contrast, PU reduced the nuclear accumulation of DOX (red fluorescence) in association with greater GSTP expression (green fluorescence) in the nucleus (Fig. 2B Lower panel). GSTP activity was evaluated at 6, 12 and 24 h after the treatments. Although the level of activity varied among groups, no significant difference was detected (Fig. 2C). Effects of PU and DOX on the expression levels of GSTP, GCS, MnSOD, and CuZnSOD were also examined and shown in Fig. 2D as representative gels. PU and DOX did not significantly increase the protein level of GSTP throughout the time course (Fig. 2D). In the presence of PU, the effect of DOX-induced GSTP expression was similar to that of without PU. Together, GSTP expression was not changed by both PU and DOX as well as the protein levels of GSTP were not statistically different between DOX and DOX plus PU, which was consistent with the results of GST activity. Other GST classes (GSTA and GSTM) were not detected at all time points (data not shown). The protein levels of γ-GCS did not show a significant change in any treated groups (Fig. 2D). In addition, expression patterns of another two important antioxidant isozymes (MnSOD and CuZnSOD) for the scavenging of superoxide were evaluated (Fig. 2D). PU slightly increased MnSOD expression at 6 h, but no significant change was detected afterwards. The expression levels of MnSOD in cells treated with DOX or DOX+PU were not strikingly altered at any time point. In addition, no significant change in the CuZnSOD isoform was found in the treatment groups.

RNA interference for GSTP increased nuclear accumulation of DOX and apoptosis

Cells transfected with GSTP SiRNA showed suppression of GSTP expression as evaluated by immunoblotting (Fig. 3A). Effects of RNA interference and PU on the accumulation of intracellular DOX were evaluated as described in the methods using flow cytometry (Fig. 3B). Suppression of GSTP expression increased DOX accumulation as early as 1 h. PU

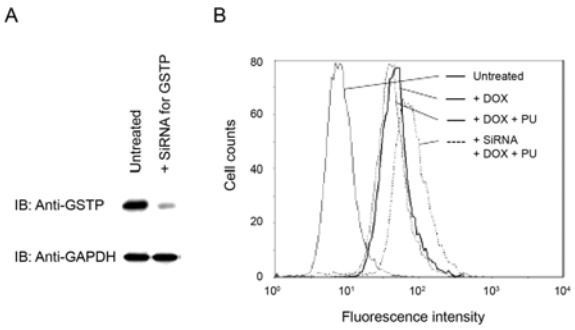

Fig. 3 Effect of RNA interference with GSTP on the expression of GSTP and intracellular DOX content in H9c2 cells. (A) H9c2 cells were transfected with or without SiRNA for GSTP as described in the methods. After 48 h, the expression level of GSTP was examined by immunoblot (IB) analysis in untransfected H9c2 cells (untreated) and cells transfected with GSTP SiRNA (SiRNA for GSTP). (B) Following 48 h transfection with or without SiRNA for GSTP, cells were treated with DOX (1 μM) plus PU (10 μg/ml) for 1 h, and the intracellular amount of DOX was evaluated by flow cytometry as described in the methods

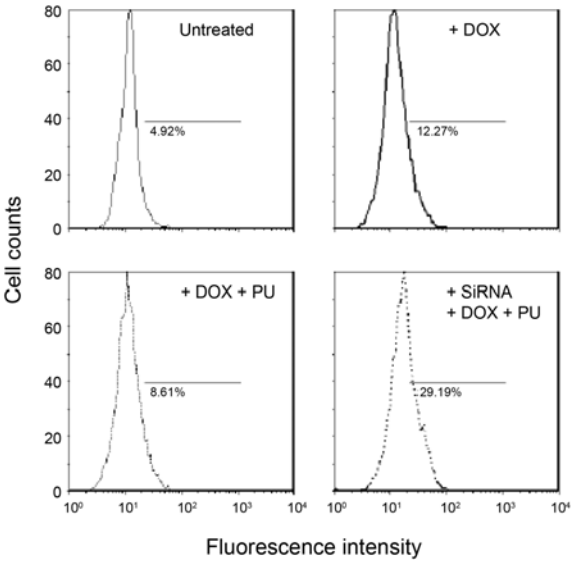

Fig. 4 Influence of RNA interference with GSTP on DOX-induced apoptosis and the antiapoptotic effect of PU in H9c2 cells. Untransfected and SiRNA-transfected H9c2 cells were treated with DOX (1 μM) plus PU (10 μg/ml) for 24 h, and the incidence of apoptosis was determined by TUNEL assay as described in the methods

decreased DOX accumulation in untransfected cells but not in SiRNA-transfected cells at 1 and 3 h of treatment. Shown in Fig. 4, apoptosis was examined by the TUNEL assay at 24 h in wild-type and SiRNA-transfected cells. Generally, apoptotic cells make up at less than 5% of a normal cell population. DOX increased proportion of cells positive with TUNEL while PU+ DOX reduced percent of apoptotic cell when compared to DOX treatment alone. PU did not show this effect when treated in SiRNA-transfected cells (data not shown). Suppression of GSTP with SiRNA markedly increased the population of TUNEL-positive cells which suggested that GSTP may have a protective role against DOX toxicity.

Discussion

GSTs are a family of enzymes crucial in the detoxification of xenobiotics and catalyzing the degradation of ROS adducts generated by a wide variety of oxidative insults, including DOX[17,26]. It is well recognized that GSTs play an important role in the resistance of cancer cells to DOX chemotherapy but the role of GSTs in prevention of DOX cardiotoxicity is not well defined[27,28]. In this study, the plant extract PU showed a unique mechanism of cytoprotection, influencing the nuclear localization of GSTP in H9c2 cells treated with DOX without significantly affecting the enzymatic activity. Suppression of GSTP expression by RNA interference potentiated the accumulation of DOX in the nucleus and enhanced apoptosis. This suggests that GSTP is required in H9c2 cells for protection against DOX.

In certain cell types, GST is activated in response to oxidative stress partially through the transcription factors AP1 and Nrf2[29,30]. GST activity can be raised up to 14-fold in DOX-resistant cancer cells relative to sensitive cells[31] or show no significant alteration[32]. Similar conflicting data exist for cardiac tissue. For example, Paranka and Dorr[33] reported that DOX time-dependently increased GST activity in cultured rat heart cells while DOX did not increase GST activity but significantly activated another detoxification enzyme, DT-diaphorase, in atria isolated from guinea pigs[34]. The regulation of GST expression and GST activity is quite complex at the level of transcription and post-transcription[13,35,36]. Our findings are in agreement with others' in that DOX increased levels of GSTP protein[37]. The measurement of GST activity is usually performed *in vitro* where its substrate glutathione (GSH) is readily available in excess amounts and so the results may not reflect the actual enzyme activity *in vivo* or in live cells. Nevertheless, our results indicated that the nuclear localization of GSTP plays a more significant role in cytoprotection than an increase in enzyme activity. Despite many intracellular components are targets of DOX, evidence from several studies has shown that the nuclear concentration of DOX is a determinant of its cytotoxicity[2,38,39]. Therefore, the nuclear localization of GSTP may reduce the accumulation of DOX hence protecting cells against DOX-induced DNA damage.

It is well established that DOX-induced apoptosis is associated with the fragmentation of DNA, release of cytochrome c, and generation of ROS. The plant extract PU suppressed apoptosis by decreasing nuclear DOX accumulation and presumably inhibiting cytochrome c release by enhancing MnSOD expression. This activation along with an increase in GSH content[10] may contribute to the anti-apoptotic effect observed in SiRNA-transfected H9c2 cells. In knockout mice, the loss of certain GST isoenzymes causes an up regulation in the expression of the remaining transferases[13], but in this study we did not observe any up regulation of other GST classes (GSTA and GSTM). This could be because the study was not long enough to observe the change or there was no switch in phenotypes in this cell line.

The role of GST is not limited to phase II detoxification enzyme that catalyze the conjugation of toxic metabolites with GSH but it also involves in modulation of apoptotic pathways in favor of cyto-protection. For example, GSTM and GSTP inhibited stress signal mediated by ASK1/JNK-p38 pathway and this activity is not dependent on catalytic activity in intracellular glutathione metabolism[40-42]. In this study, it is possible that the mechanisms of PU-mediated cytoprotection may occur through the change in nuclear localization of GSTP as well as modulation of ASK1/JNK activities. However, further study is warranted.

Rat cardiacmyoblast H9c2 have been used extensively as a model to study the cardiotoxicity of DOX and oxidative damage in cardiac tissues[24,43,44]. However, the endogenous expression pattern of GST classes in this cell line has not been reported. It appears that GSTP is the major class of GST present in H9c2 cells although increased GSTA mRNA expression (no immunoblot evidence) has been detected[45]. Thus, our study provides useful information for studies of oxidative-related antioxidant enzymes such as GST in H9c2 cells.

Conclusion

In summary, our study demonstrated that GSTP played an important role in cytoprotection against DOX and this effect specifically reduced the nuclear accumulation of DOX at a very early point of time. Other antioxidant enzymes are also important in oxidative stress, but their functions are more universal. PU suppressed DOX-induced apoptosis via mechanisms that involved the nuclear localization of GSTP, leading to the activation of endogenous antioxidant defenses. It is important to understand the basis/characteristics of oxidative mechanisms in order to search for strategies to protect cells against different kinds of oxidative insults. Thus, plant extracts may be alternative sources of antioxidants with unique mechanisms of action that may be suitable for specific oxidative-related pathologies.

Acknowledgments

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บทบาทของ Phyllanthus urinaria L. และ Glutathione-S transferase Pi ในการปกป้องความเป็นพิษต่อเซลล์กล้ามเนื้อหัวใจชนิด H9c2 ที่เกิดจากการเหนี่ยวนำของยา doxorubicin

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***วัตถุประสงค์**: เพื่อศึกษาฤทธิ์ปกป้องเซลล์ของสารสกัดเอธานอลของหญ้าใต้ใบ (Phyllanthus urinaria, PU) ต่อความเป็นพิษที่เกิดจากยา doxorubicin (DOX) โดยเน้นถึงกลไกการออกฤทธิ์ที่สัมพันธ์กับการแสดงออกของยีนและการกระจายตัวของ glutathione-S transferase (GST) ในเซลล์หัวใจชนิด H9c2*

***วัสดุและวิธีการ**: ศึกษาการแสดงออกของ GST isoforms ในเซลล์ H9c2 ด้วยวิธี western blot analysis และ confocal immunofluorescence visualization เซลล์ที่ได้รับ DOX ถูกแบ่งเป็นสองกลุ่มคือ ในภาวะที่มี PU และไม่มี PU แล้วตรวจหาตัวบ่งชี้ถึงฤทธิ์ปกป้องเซลล์ ได้แก่ การแสดงออกของ γ-glutamylcysteine synthetase (γ-GCS), manganese superoxide dismutase (MnSOD), copper-zinc SOD (CuZnSOD), และการทำงานของ GST ที่ได้จาก cell lysate มีการศึกษาการมีส่วนร่วมของ GST ในการปกป้องเซลล์ โดยใช้เทคนิค RNA interference และตรวจ วัดการเกิด apoptosis โดยใช้ TUNEL assay*

***ผลการศึกษา**: GST Pi (GSTP) เป็นชนิดที่พบมากที่สุดในเซลล์ชนิด H9c2 เมื่อเปรียบเทียบกับ GST Alpha (GSTA) และ GST Mu (GSTM) การให้ PU ช่วยป้องกันความเป็นพิษของเซลล์ที่เกิดจากการเหนี่ยวนำของ DOX โดยผ่านกระบวนการเคลื่อนที่ของ GSTP เข้าสู่นิวเคลียสโดยไม่มีผลกระทบอย่างมีนัยสำคัญต่อการทำงานของเอนไซม์ GST การกดการแสดงออกของ GSTP โดยใช้ RNA interference ทำให้เพิ่มการสะสมของ DOX ในนิวเคลียสของเซลล์ และเพิ่มการเกิด apoptosis เมื่อตรวจวัดโดย TUNEL assay การให้ PU มีฤทธิ์ปกป้องเซลล์จากการลดปริมาณของ DOX และเพิ่มการกระจายของ GSTP เข้าสู่นิวเคลียสของเซลล์กล้ามเนื้อหัวใจ*

***สรุป**: กลไกการปกป้องเซลล์ของ PU ที่เกิดจาก DOX มีส่วนเกี่ยวข้องกับการแสดงออกของ GSTP ดังนั้นสารสกัดจากหญ้าใต้ใบจึงอาจเป็นแหล่งหนึ่งของสาร antioxidants ที่มีกลไกการออกฤทธิ์จำเพาะและเหมาะกับ oxidative insults บางชนิด*