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ORIGINAL RESEARCH article

Modulation of TCDD-induced Cyp1a1 mRNA by the Co-repressor SMRT

Issa E.A. Amara [1] and **Shaban E.A. Saad** [2 *]

[1] Department of Pharmacology, Faculty of Medicine, University of Zintan, Zintan, Libya,
[2] Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, University of Tripoli, Tripoli, Libya
[*]Author to whom correspondence should be addressed

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Abstract: Environmental factors are known to influence carcinogenesis. Pollutants, such as heterocyclic amines (HAs) and polycyclic aromatic hydrocarbons (PAHs) are examples of environmentally borne procarcinogens. Procarcinogens are not active carcinogens although, they require bio-activation, via enzymes such as cytochrome P450 (CYP) to transform into the active products. CYP1A1 isoform, regulated by aryl hydrocarbon receptor (AhR) plays a significant role in the bio-activation of PAHs and HAs. AhR has multiple co-activators and co-repressors for AhR that have been identified S remarkably. Silencing mediator for retinoid and thyroid hormone receptors (SMRT) were designated as a major co-repressor for AhR. The present study is examined the effect of SMRT overexpression on the cyp1a1 mRNA levels in murine hepatoma Hepa 1c1c7 cell line. Hepa 1c1c7 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) and competent cells have successfully been produced using the calcium chloride method. Cells were transformed using plasmid DNA and Lipofectamine. TCDD (2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin) was employed as an inducing agent for CYP1A1. The total cellular RNA was isolated and real-time PCR of Cyp1a1 was conducted. A statistical analysis was conducted by using a one-way analysis of variance followed by the Student-Newman-Keuls test. The results showed that cells transfected with the co-repressor SMRT had lower TCDD-mediated induction of cyp1a1 mRNA without affecting constitutive Cyp1a1 mRNA levels, i.e., SMRT was able to significantly decrease inducible Cyp1a1 mRNA levels in Hepa 1c1c7 cells. The present findings also demonstrated that calcium chloride is a convenient method for routine transformation. Thus, this study opens a new avenue for the management of carcinogenesis involving CYP1A1-inducing carcinogens.

Introduction

Numerous studies have supported the hypothesis that environmental factors induce the process of carcinogenesis [1]. Among these factors, procarcinogens as heterocyclic amines (HAs) and polycyclic aromatic hydrocarbons (PAHs), are chemically inert and require bio-activation into electrophilic metabolites to exert their genotoxic effects which is generally localized at the site of expression of bio-activation enzymes.

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Cytochromes P450 (CYPs) metabolizes a wide range of the compounds of endogenous and exogenous origin, in addition it plays a major role in the bio-activation processes of these procarcinogens [2-4]. In humans, CYP1A1 isoform, is expressed in extra-hepatic tissues [5] which is largely involved in the bio-activation of PAHs and HAs. Variation of CYP1A1 expression can consequently modulate cell susceptibility to procarcinogen exposures. CYP1A1 is regulated by a transcription factor, the aryl hydrocarbon receptor (AhR) [6, 7]. The AhR is a member of the family of basic-helix-loop-helix/Per-Arnt-Sim (bHHLH/PAS) transcription factors [8].

AhR is a cytosolic protein with a molecular weight of about 88 kDa [9]. The receptor is made up of several functional domains such as those for ligand binding, DNA binding, dimerization, transactivation, nuclear import/export and heat shock protein 90 (Hsp90) interaction [9]. Furthermore, the AhR is a ligand-dependent transcription factor that regulates the expression of a wide array of genes in many species and tissues [10]. Following ligand binding in the cytoplasm, AhR is internalized and dimerized with the aryl hydrocarbon receptor nuclear translocator (Arnt). This heterodimer acquires the ability to interact with xenobiotic-responsive elements (XREs) and enhances the transcription of CYP1A1 gene.

Moreover, low doses of retinoic acid (RA), a natural vitamin A metabolite, slightly induce cutaneous CYP1A1 expression by activation of the nuclear receptor (NR) RAR (RA receptor). The RAR-RXR (retinoid X receptor) heterodimer binds the RA-responsive element sequence located within the CYP1A1 promoter [11]. Many carotenoids (α- and β- carotene, β-cryptoxanthin), contained in food, can be metabolized into products with retinoid activity and activate CYP1A1 through this signaling pathway [12]. Several studies have demonstrated that the activity of the NR superfamily members is dependent on the interactions with other proteins, including a large number of co-activators and co-repressors that are important tissue/cell-specific mediators of the NR functions [13, 14]. Indeed, unliganded RAR can interact with two different co-repressors, N-CoR (nuclear receptor co-repressor) or SMRT (silencing mediator for retinoid and thyroid hormone receptors) [15] which form large protein complexes including histone deacetylases (HDACs). These HDACs will then specifically affect the accessibility and/or function of transcriptional regulatory proteins that bind DNA sequences and subsequently trigger inhibition of target genes [16]. The regulation of CYP1A1 gene expression by single compounds was extensively investigated. Nevertheless, it is constantly exposed to a set of environmental pollutants and little is known about the effects of exposure to different inducers acting on different receptors like AhR and RAR. Despite of a structurally diverse group of ligands, the prototypical AhR ligand is the anthropogenic contaminant 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) [8]. TCDD is the most potent inducer with the greatest affinity towards AhR [17]. Thus, TCDD was used as an inducer for Cyp1a1 mRNA which reflects the activation of the AhR.

Although activation of transcription has long been recognized as an essential component of gene regulation, the realization that repression of the transcription plays a fundamental role has been appreciated. Numerous studies have identified several nuclear proteins that modulate transactivation by members of the nuclear receptor superfamily of transcription factors that include the steroid and thyroid hormone, vitamin D and retinoid receptors and several orphan receptors. More, physical and functional interactions between AhR/Arnt and co-activators such as estrogen receptor associating protein 140 (ERAP 140) and co-repressors such as SMRT were studied. Both ERAP 140 and SMRT physically interact with AhR/Arnt and have shown an increase in the interaction between AhR/Arnt-dioxin response element (DRE) by ERAP, but this interaction was decreased by SMRT as evident by electrophoretic mobility shift assays (EMSA) [14]. Similar results have shown that the nuclear receptor co-activator (ERAP140) increased the transcriptional activation mediated by AhR/Arnt while the co-repressors (SMRT) was able to decrease it in MCF-7 human breast cancer cells [14]. Because of these

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results, the co-activator was identified as the protein that increases gene expression by binding to an activator (or transcription factor) which contains the DNA binding domain while being unable to bind DNA by itself [18-20]. On the other hand, the co-repressor is a protein that decreases gene expression by binding to a transcription factor that contains a DNA binding domain and is unable to bind DNA by itself as well. The co-repressor can repress transcriptional initiation by recruiting histone deacetylases which catalyze the removal of acetyl groups from lysine residues. This, in turn, increases the positive charge on histones which strengthens the interaction between the histones and DNA, making the later less accessible to transcription [21, 22]. This study, is focused on the co-repressor protein that depends, at least in part, on the activities of histone deacetylases (HDACs) such as SMRT. Also, it is assumed that SMRT, as a co-repressor for AhR, decreases the TCDD-mediated induction of AhR-regulated genes, typified by Cyp1a1, by inhibiting their transcriptional expression. Thus, it aims to examine the effect of SMRT over expression on Cyp1a1 mRNA levels in murine hepatoma Hepa 1c1c7 cells.

Materials and methods

Chemicals: Bacto-tryptone and bacto-yeast extract were from Bacton Dickinson and company Sparks (MD, USA). 2, 3, 7, 8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was from Cambridge Isotope Laboratories (Woburn, MA, USA). Ampicillin sodium salt from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Select agar, lipofectamine 2000 and calcium phosphate transfection kits from Invitrogen Co. (Grand Island, NY, USA). pCMX-mSMRTa FL plasmid DNA was provided by Ronald M. Evans (Howard Hughes Medical Institute Research Laboratories, USA). All the other chemicals from Fisher Scientific Co. (Toronto, Canada).

Cell culture: Bacteria (*E. coli* top 10) have generously been provided by Suresh Mavanur (University of Alberta, Canada). Hepa 1c1c7 cell line, ATCC number CRL-2026 (Manassas, VA) was maintained in Dulbecco's modified Eagle's medium (DMEM) without phenol red, added with 10.0% heat inactivated fetal bovine serum, 20 µM l-glutamine, 50 µg per ml amikacin, 100 IU per ml penicillin, 10 µg per ml streptomycin, 25 ng per ml amphotericin B, 0.10 mM non-essential amino acids and vitamin supplement solution. Cells were grown in 75-cm^2 cell culture flasks at 37^0C in a 5.0% CO_2 humidified incubator.

Preparation and transformation of competent cells: Competent cells have successfully been done using the calcium chloride method. Competency is the physiologic state that changes the structure and permeability of the cell membrane, so the plasmid DNA can enter the cell. *E. coli* cells are grown to log phase. Then, the cells are concentrated by centrifugation and re-suspended in a solution containing calcium chloride. Exposure to calcium ions renders the cells able to take up DNA, or competent. Plasmid DNA is mixed with the cells and presumably adheres to them. The mixture of DNA and cells is then heat shocked which allows the DNA to efficiently enter the cells. The cells are grown in non-selective medium to allow synthesis of plasmid-encoded antibiotic resistance proteins and plated on antibiotic-containing medium to allow identification of plasmid-containing colonies.

DNA isolation from E. coli plasmid pCMX-mSMRTα FL: pCMX-mSMRTα FL stock (-20^0C) was thawed to room temperature and 15 µl was incubated in 500 ml Luria-Bertani (LB) medium which was prepared by dissolving 10.0 g Bacto-Tryptone, 5.0 g Bacto-yeast extract, 5.0 g glucose and 10.0 g NaCl in one liter of double-deionized H_2O. Ampicillin (50 mg per ml) was added immediately before growing culture. A starter culture of 3.0 ml LB medium was inoculated by picking a single colony from a freshly streaked selective plate and incubated for 8.0 hours at 37^0C with vigorous shaking at a speed of 300 rpm. The culture was diluted one per 500 into 3.0 ml LB medium. Cells were grown at 37^0C for 16 hours with vigorous shaking at a speed of 300

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rpm. Cells were then pelleted and isolation of DNA was done using Quiagen plasmid purification kit. Briefly, the cells pellet was placed in an alkaline lysis solution containing RNase A with continuous vigorous shaking to ensure complete lysis. Following that, centrifugation was done at 14,000 rpm for 10 min at 4^0C to precipitate the cell debris and proteins. The supernatant containing the plasmid DNA was then passed through Quiagen-tip. The adsorbed DNA was then washed several times before being eluted and subsequently precipitated by isopropanol followed by immediate centrifugation at 12,000 rpm for 30 min. The DNA pellet was dissolved in an appropriate volume of autoclaved double-deionized H_2O and the concentration was measured using a U.V. Spectro-photometer at a wave-length of 260 nm.

DNA detection using ethidium-agarose gel: Sample aliquots from different isolation and purification steps of plasmid DNA were run in ethidium-agarose gel. Simply, agarose gel in the concentration of 1.5% was prepared in 1xTAE (Tris-HCl acidic acid EDTA) buffer added to 7.5 µl of 10 mg per ml ethidium bromide stock. Samples were run on 90 volts for 2 hours followed by visualization using UV transluminator and digitally recorded using Gel Doc-It imaging system, UVP Bioimaging system (Upland, CA).

Transfection using lipofectamine 2000: Transfection has successfully been carried out using manufacturer instructions. Firstly, murine hepatoma Hepa 1c1c7 cells were seeded in a 12-well plate. After the 24-hour the medium was changed to serum-free, antibiotics-free medium while preparing the transfection mixture. For each well, 1.6 µg DNA in 100 µl medium, (tube A) and 4.0 µl lipofectamine in 100 µl medium (tube B) were prepared. Both tubes were incubated together for 20 min before adding to the well. Cells were then incubated for at least 18 hours before treatment with SFM (serum free medium) or one nM TCDD for 6 hours for normal and transfected cells.

RNA extraction and quantitative real-time PCR of cyp1a1: After incubation with the test compounds for the specified periods, total cellular RNA was isolated using TRIzol reagent, according to the manufacturer's instructions (Invitrogen) and quantified by measuring the absorbance at 260 nm. For reverse transcription-polymerase chain reaction (RT-PCR), first-strand cDNA was synthesized from 1.0 µg of total RNA using the high-capacity cDNA reverse transcription Kit with random primers. Real-time PCR reactions were performed on an ABI 7,500 real-time PCR system (Applied Bio-systems), using SYBR® Green PCR Master Mix (Applied Bio-systems). The amplification reactions were performed as follows: 10 min at 95^0C and 40 cycles of 94^0C for 15 sec and 60^0C for one min. Primers and probes for mouse Cyp1a1 were: Forward primer 5`- GGT TAA CCA TGA CCG GGA ACT -3`, reverse primer 5`- TGC CCA AAC CAA AGA GAG TGA -3` and for β-actin: forward primer 5`- TAT TGG CAA CGA GCG GTT CC -3`, reverse primer 5`- GGC ATA GAG GTC TTT ACG GAT GTC -3` were purchased from integrated DNA technologies (IDT, Coralville, IA). The fold change in the level of yp1a1 (target gene) between treated and untreated cells, corrected by the level of ß-actin, was determined using the following equation: Fold change=2-Δ(ΔCt), where ΔCt=Ct(target)-Ct(ß-actin) and Δ(ΔCt) =ΔCt(treated)-ΔCt(untreated).

Statistical analysis: The comparative analysis of the results from various experimental groups with their corresponding controls was performed using SigmaStat for Windows (Systat Software, Inc, CA). One-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test was carried out to assess which treatment groups showed a significant difference from the control group. The difference level was considered significant when $p<0.05$.

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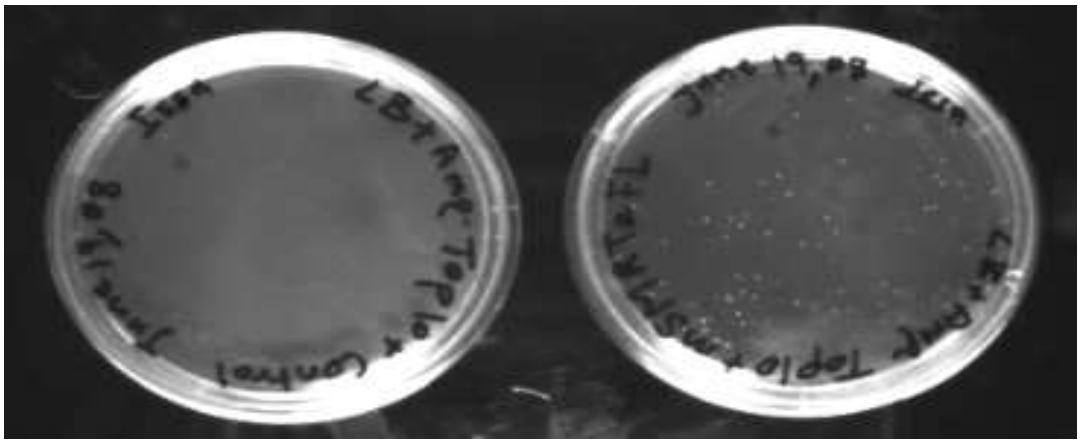
Results

Preparation and transformation of competent cells: Plasmid-containing colonies were identified in agar plates containing ampicillin for selective growing. The colonies appeared clearly in plate containing transformed cells and, on the other hand, there were no colonies in the plate containing untransformed *E. coli* (**Figure 1**).

Figure 1: Cells without and without DNA plasmid



A **B**

Plate A: cells without DNA plasmid and Plate B: cells transformed with plasmid DNA, where the colonies appear clearly

DNA isolation using Quiagen plasmid purification kit: In **Figure 2**, four samples from different purification steps were run on 1.5% ethedium-agarose gel (samples 1 to 4). Sample 1 is the crude lysate containing DNA and RNA contaminants. Sample 2 was collected after the crude extract was run on the Quiagen-tip resin; thus, it had smaller amounts of DNA but also contained RNA. Sample 3 is an aliquot of washing steps to remove the RNA from the resin and thus the sample had no DNA. Sample 4 which is of a great importance, as it is the eluate that contains the DNA bound to the beads, had a relatively light band as it was diluted with QF buffer. This eluate was then precipitated using isopropanol and the resultant DNA pellet was dissolved in DNase/RNase-free water before running in the last lane (DNA lane).

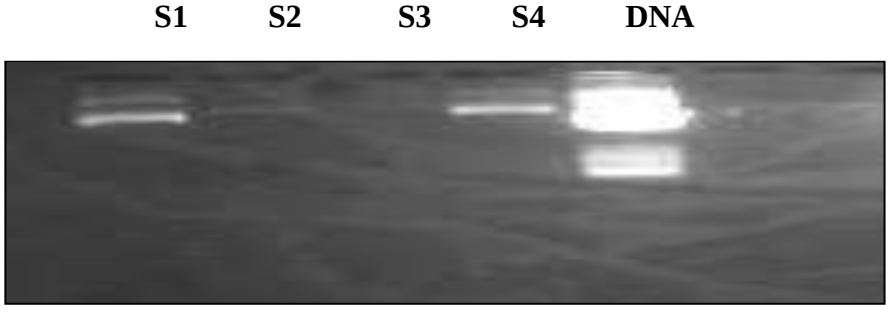


Figure 2: Detection of DNA using ethidium-agarose gel.

Effect of SMRT on Cyp1a1 in Hepa 1c1c7 cells: In order to understand the kinetics of cyp1a1 in response to the co-repressor SMRT and TCDD, hepa1c1c7 cells transiently transfected with SMRT were treated with one nM TCDD (**Figure 3**). Thereafter, cyp1a1 mRNA was assessed using real-time PCR. The results showed that cells transfected with SMRT did not possess a significant difference than that of the control. Furthermore, transiently

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transfected cells treated with TCDD have shown a significant decrease in the cyp1a1 mRNA levels compared to the TCDD treatment in normal cells. These results imply that transfection with the co-repressor SMRT decreases TCDD-mediated induction of cyp1a1 mRNA without affecting constitutive cyp1a1 mRNA levels (**Figure 3**).

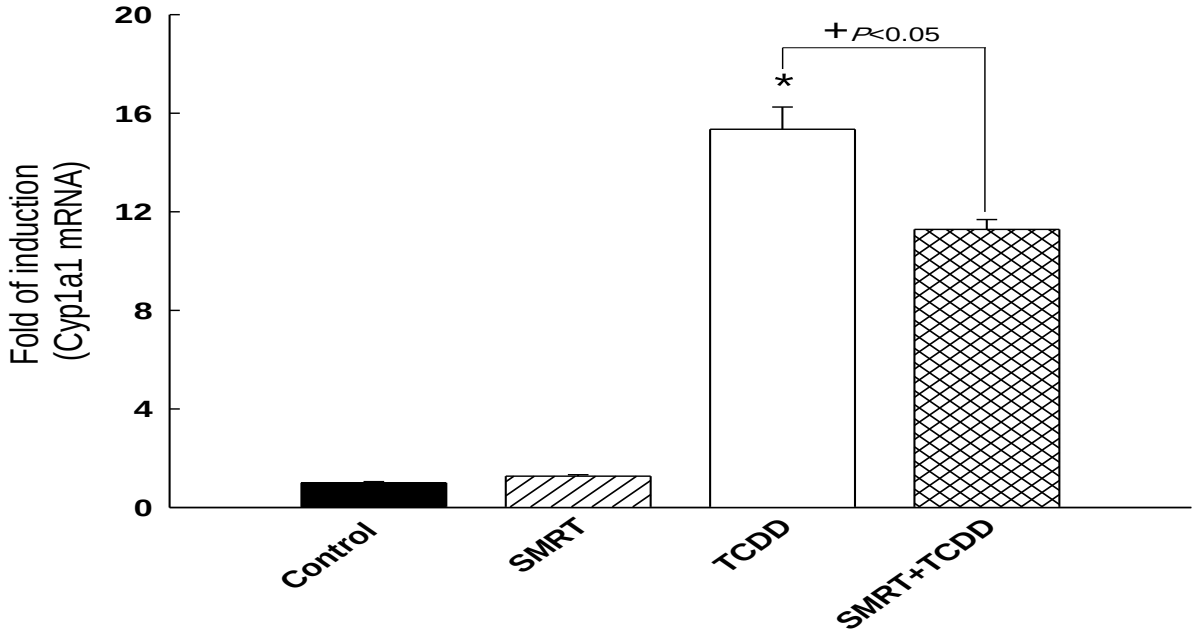

Figure 3: Effect of SMRT on TCDD-mediated induction on cyp1a1 transcriptional gene activity. No effect when SMRT gives alone, $P<0.05$ *vs* control

Discussion

Although, we did not make a comparison study between the methods that induce cell competence, *E-coli* transformation efficiency has greatly been enhanced, making calcium chloride method ideal for the routine transformations. The presence of colonies in the antibiotic agar plate indicates that the competent preparation steps for cells and the transformation of DNA plasmid were success. This study aimed to perform the competent cells using calcium chloride method and to introduce pCMX-mSMRTα FL plasmid DNA into hepa 1c1c7 cells as previously mentioned. The second objective was to observe the effect of overexpression of the co-repressor protein SMRT on the induction of cyp1a1 mRNA by TCDD. Thus, there are several distinct mechanisms by which the co-repressor proteins induce their effects, including competition with activator proteins for DNA binding, sequestration of such activators, interaction with core transcriptional machinery, DNA methylation and recruitment of complexes that have histone deacetylase activity [16]. In this context, it focuses on the co-repressor proteins that utilize the actions of histone deacetylases to exert their effect such as SMRT [15, 23-25]. Nuclear receptors bound to XRE recruit significant number of other proteins (referred to transcription co-regulators) which facilitate or inhibit the transcription of the associated target gene into mRNA [26, 27]. The functions of co-regulators are varied and include chromatin remodeling by making the target gene either more or less accessible to transcription or bridging to stabilize the binding of other co-regulatory proteins. The binding of agonist ligands such as TCDD to nuclear receptors induces a conformation of the receptor that

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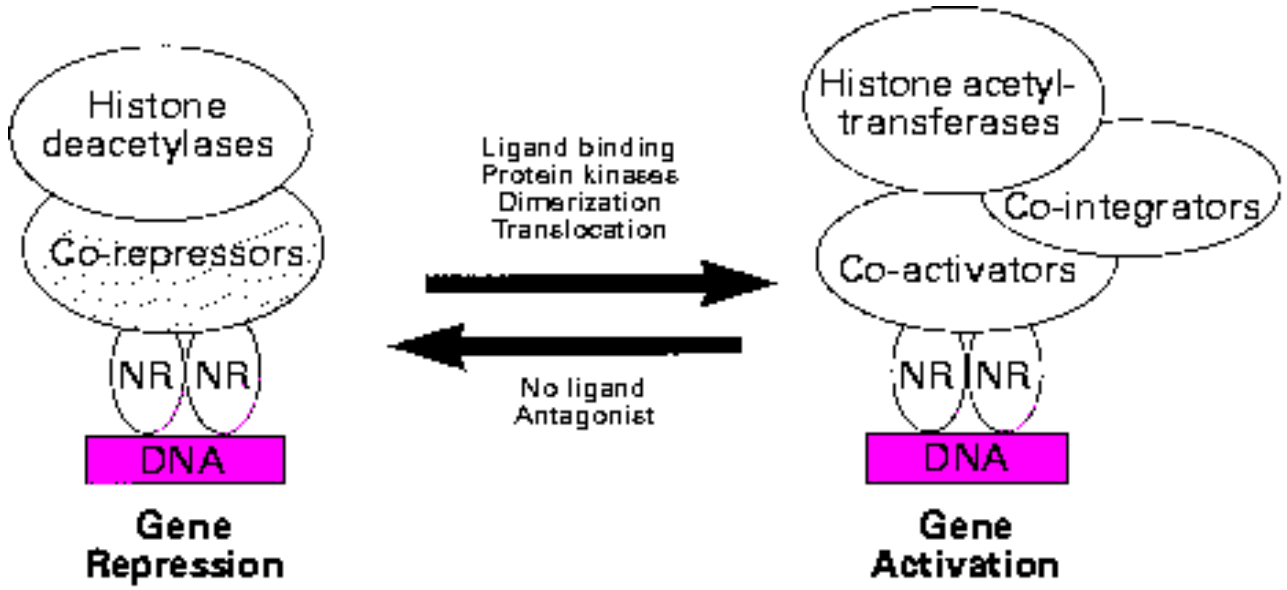
preferentially binds co-activator proteins. These proteins often have an intrinsic histone acetyltransferase (HAT) activity which weakens the association of histone to DNA and therefore promotes gene transcription. However, in the presence of co-repressor proteins such as SMRT which is in turn recruits histone deacetylases (HDACs) which strengthens the association of histone to DNA and then represses gene transcription (**Figure 4**).

Figure 4: Mechanism of repression via deacetylation of nuclear receptor



A **B**

A: Ligand agonist recruits transcriptional co-activators such as histone acetyltransferase proteins for transcriptional co-activator after its binding to nuclear receptor (AhR). **B**: Co-repressor proteins such as SMRT in turn recruit's histone deacetylases proteins for transcriptional repression.

The closely related co-repressor proteins nuclear receptor co-repressor (N-CoR) and SMRT have been shown to be recruited to many classes of transcription factor and are in fact components of multiple protein complexes containing deacetylase proteins. This association with the HDAC activity provides an important component of the mechanism that allows DNA-binding protein interact with N-CoR or SMRT to repress transcription of specific target genes [16]. In addition, the understanding of the biological role of HDAC particularly, HDAC3 came with the realization that the enzyme forms a stable complex with N-CoR and SMRT. Identification of HDAC3 as the catalytic component of N-CoR/SMRT complexes provided a mechanistic link between transcriptional repression and histone deacetylation. Via their interactions with a number of different transcription factors, the two co-repressors recruit HDAC3 to specific promoters where the enzyme deacetylases histones and mediates the silencing of the corresponding genes. Furthermore, histone deacetylation is thought to repress gene expression by at least two different mechanisms. First, removal of the acetyl group increases the local positive charge of histones, increasing their affinity for the negatively charged DNA. This generates a tight chromatin structure, refractory to transcription. Moreover, deacetylation reduces the affinity of bromo-domain-containing co-activators [28]. In this way, the N-CoR/SMRT complex mediates the repression of unliganded nuclear receptors such as TR and RAR [23, 24]. Generally, unliganded receptor associates with N-CoR/SMRT, but this interaction is lost as a result of the receptor conformational change upon ligand binding such as TCDD [28, 29]. Although, some studies reported that the precise roles of histone deacetylation in transcriptional repression are not fully understood, Karagianni and Wong [28] suggested that a growing of non-histone

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substrates extends the role of HDAC3 beyond transcriptional repression. Nguyen and co-workers [13] reported a direct interaction of the SMRT with the AhR and this interaction resulted in a decrease in AhR:ARNT:DNA complex formation and AhR-dependent gene expression. The findings of the current study demonstrate an inhibitory effect of SMRT on TCDD-induction mediated gene cyp1a1 mRNA levels in hepa 1c1c7 cells. This inhibition may be in part due to the removal of the acetyl group from histones which is increases it's binding to DNA making the later less accessible to transcription. More, histone deacetylation reduces the affinity of bromo-domain-containing co-activators [28].

Conclusion: For the preparation of competent cells, calcium chloride is a convenient method for routine transformation. The obtained data from the effects of SMRT on TCDD-mediated induction revealed that SMRT is able to significantly decrease inducible Cyp1a1 mRNA level in Hepa 1c1c7 cells.

Author's contribution: IEAA conceived, designed the study, performed the analysis and drafted the manuscript. SEAS contributed to drafting the manuscript. Both authors have approved the final version of the manuscript and agreed to be accountable for its contents.
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Ethical issues: Including plagiarism, informed consent, data fabrication or falsification and double publication or submission were completely observed by the authors.
Data availability statement: The raw data that support the findings of this article are available from the corresponding author upon reasonable request.
Author declarations: The authors confirm that all relevant ethical guidelines have been followed and any necessary IRB and/or ethics committee approvals have been obtained.

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