Original article

In vivo and in vitro studies evaluating the chemopreventive effect of metformin on the aryl hydrocarbon receptor-mediated breast carcinogenesis

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ABSTRACT

Metformin (MET) is a clinically used anti-hyperglycemic agent that shows activities against chemically-induced animal models of cancer. A study from our laboratory showed that MET protectes against 7, 12-dimethylbenz[a]anthracene (DMBA)-induced carcinogenesis in vitro human non-cancerous epithelial breast cells (MCF10A) via activation of the aryl hydrocarbon receptor (AhR). However, it is unclear whether MET can prevent the initiation of breast carcinogenesis in an in vivo rat model of AhR-induced breast carcinogenesis. Therefore, the main aims of this study are to examine the effect of MET on protecting against rat breast carcinogenesis induced by DMBA and to explore whether this effect is medicated through the AhR pathway. In this study, treatment of female rats with DMBA initiated breast carcinogenesis though inhibiting apoptosis and tumor suppressor genes while inducing oxidative DNA damage and cell cycle proliferative markers. This effect was associated with activation of AhR and its downstream target genes; cytochrome P4501A1 (CYP1A1) and CYP1B1. Importantly, MET treatment protected against DMBA-induced breast carcinogenesis by restoring DMBA effects on apoptosis, tumor suppressor genes, DNA damage, and cell proliferation. Mechanistically using in vitro human breast cancer MCF-7 cells, MET inhibited breast cancer stem cells spheroids formation and development by DMBA, which was accompanied by a proportional inhibition in CYP1A1 gene expression. In conclusion, the study reports evidence that MET is an effective chemopreventive therapy for breast cancer by inhibiting the activation of CYP1A1/CYP1B1 pathway in vivo rat model.

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1. Introduction

Breast cancer is one of the most commonly present cancers in females with approximately 2.09 million cases in 2018 (Bray et al., 2018). Breast tumors are also the second most common causes of death, where 626,679 women die due to cancer every year (Papadakis et al., 2021). The high relapse rate and treatment failure of breast cancer due to chemoresistance encourage researchers to identify new natural and synthetic cancer chemopreventive agents. According to recent statistics about breast cancer, it was reported that 85% of cancer cases are be attributed genetic mutations due to the aging process, lifestyle changes, and environmental factors (White et al., 2018). Among these factors, exposure to environmental pollutants such as 7,12-dimethylbenz[a] anthracene (DMBA) and 2,3,7,8-tetrachlorodibenzo[p]dioxin (TCDD), common polycyclic aromatic hydrocarbons (PAHs), inducing a powerful organ-specific laboratory breast carcinogenesis (Ferreira et al., 2013; Ip, 1996). It is well documented that the ability of DMBA and TCDD to induce organ carcinogenesis is mediated through the activation of a cytosolic transcription factor, the aryl hydrocarbon receptor (AhR) (Kerzee and Ramos, 2001; Whitelaw et al., 1993).

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Upon binding to its ligands such as DMBA and TCDD, AhR enters the nucleus to bind to its nuclear translocator (ARNT) (Pollenz, 2002). This dimer targets the xenobiotic responsive element (XRE) on the enhancer region of several xenobiotic metabolizing enzymes, including the cytochrome P450 1A1 (CYP1A1) and CYP1B1 to initiate of transcription and translation process. Transcriptional induction of these genes results in the bioactivation of procarcinogens into their ultimate carcinogens through the formation of DNA adduct leading to cancer initiation (Whitlock, 1999). The involvement of AhR/CYP1 pathway in cancer initiation and organ carcinogenesis and tumorigenesis is evidenced by the findings that AhR activation causes increases in the proliferation of human breast cancer cells and their cancer stem cells (CSCs) (Jung et al., 2011) via the suppression of apoptosis (Bekki et al., 2015). However, the inactivation of AhR/CYP1 pathway blocks breast carcinogenesis and DNA adduct formation in vitro human epithelial breast MCF10A cells (Maayah et al., 2015). These results were supported by the observations that cyp1a-null mice, but not the wild type, were resistant to DMBA-induced breast carcinogenesis (Buters et al., 1999).

Recent studies have demonstrated that metformin (MET), an oral anti-hyperglycemic drug, exhibits anti-cancer activity against animal models of chemical carcinogenesis and cell lines of different types of cancer (Evans et al., 2005). A population-based retrospective cohort study showed that MET-treated patients with diabetes had a lower cancer-associated mortality rate than diabetics treated with either sulfonylurea or insulin (Bowker et al., 2006). A previous work from our laboratory and others has shown that MET inhibited the activity and expression of AhR/CYP1A1 pathway, which protected the human epithelial breast MCF10A cells against DMBA-initiated breast carcinogenesis (Do et al., 2014; Maayah et al., 2015). Although most of the studies on the chemotherapeutic effect of MET were conducted using in vitro cell lines (Falah et al., 2017; Thompson et al., 2015), there is still a gap in the knowledge of the chemopreventive effect of MET on AhR-induced breast carcinogenesis in vivo rat animal model. Thus, the main aims of this study were to explore the potential chemopreventive effect of MET against DMBA-induced breast cancer initiation in vivo rat model and the role of AhR/CYP1 pathway.

2. Materials and methods

2.1. Materials

Metformin, DMBA and TCDD were obtained from Toronto Research Chemicals (Toronto, ON, Canada). TRIzol, cDNA Reverse Transcription kit, SYBR Green, and all RT-PCR reagents were obtained from ThermoScientific (Foster City, CA, USA). Western blot reagents, detection kits, and acrylamide/bisacrylamide were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Primary and secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). All other chemicals were purchased from Fisher Scientific Co. (Toronto, ON, Canada).

2.2. Animal experimental design and treatment protocol

Forty female Wistar albino (WA) rats provided by the King Saud University (KSU) Animal Care Center were housed in hygienic cages at 25 °C and with a 12-h light /12-h dark cycle. The rats were randomly distributed into four groups of 10 rats each. Group I was labeled as untreated control. Rats in group II (DMBA group) received a single dose of DMBA 25 mg/kg BW S.C. in 0.1–0.2 mL sesame oil injected into the mammary glands (Al-Dhfyan et al., 2017; Chidambaram and Baradarajan, 1996). Rats in groups III (DMBA+MET) received an oral dose of MET 500 mg/kg/day

(Alhaider et al., 2011), one week prior to exposure to a single dose of DMBA and continued for additional three weeks. Rats in group V received TCDD (10 μg/kg) for three weeks, as a positive control and labeled as the TCDD group. On the 28th day, rats were anesthetized and mammary tissues were immediately extracted and then divided into two segments for RNA and protein expression studies. The animals were handled in accordance with guide for the Care and Use of Laboratory (NIH, 2011) and the study protocol was approved by the KSU Research Ethics Committee, Saudi Arabia.

2.3. Real-Time polymerase chain reaction (RT-PCR)

Total RNA from rat breast tissues and human breast cancer MCF-7 cells was extracted using the TRIzol reagent (Korashy et al., 2016a). The isolated RNA was then checked for quantity and quality using a NanoDrop® 8000 (Thermo Scientific) with an optical density 260/280 nm ratio of approximately ~ 2.0. The RNA was then reverse transcribed to its cDNA and then amplified using specific forward and reverse primers (Table 1). The mRNA expression of target genes of CYP1A1, CYP1B1, caspase-3, BAX, Forkhead box class O 3a (FoxO3a), Cyclin D1, Apurinic/apyrimidinic endonuclease 1 (APE1), and 8-Oxoguanine DNA Glycosylase (OGG1), normalized to β-actin, were quantified by RT-PCR (Applied Biosystems®) using SYBR Green Master mix. The fold change in the level of target mRNAs was calculated using ΔΔCT method (Livak and Schmittgen, 2001).

2.4. Western blot analysis

Total protein from rat breast tissue homogenates was isolated as reported previously (Abrams et al., 2003) and quantified using Direct Detect® infrared spectrophotometer (Millipore, MA, USA). Western blot analysis was conducted as reported before (Al-Dhfyan et al., 2017). About 30 μg of proteins from all animal groups were separated on 10–12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and then electrophoretically transferred to PVDF membrane. The membranes were incubated for 1 h in blocking solution followed by overnight incubation at 4 °C with specific primary antibodies against target proteins and then secondary antibodies at room temperature. The bands of the target proteins were visualized by C-DiGit® Blot Scanner (LI-COR Biosciences, USA) and then semi-quantified by ImageJ (Rueden et al., 2017).

2.5. In vitro cell culture and treatment

Estrogen receptor expressing human breast cancer cell line (MCF-7) was obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were cultured and maintained in Gibco® DMEM 4.5 g/l glucose + Glutamax, provided with 10% fetal bovine serum (FBS) and 1× antibiotic/antimycotic. The cells were grown in 75 cm² cell culture flasks in humidified incubator at 37 °C and 5% CO₂. The cells were then plated in 6-well cell culture plates for subsequent studies; RT-PCR and mammospheres formation assays.

2.6. Mammospheres formation assay

One of the methods to isolate and enrich breast CSCs is to grow cells in non-adherent, non-differentiating media which results in spherical colonies of cells that are termed as "mammospheres" (Al-Dhfyan et al., 2017). MCF-7 cells were incubated with either 0.1% DMSO control media or media containing DMBA (5 μM) in the presence and absence of MET (4 mM) for three days. The cells were then cultured in Nunclon Sphera T75 cell culture flask in the presence of StemFlex basal medium, provided with 10X StemFlex

Table 1
Rat and human primer sequences used for teal-time polymerase chain (RT-PCR) reactions.

Gene	5′→3′ Forward primer	5′→3′ Reverse primer	References
rCASPASE-3	GAGTGCTCGCAGCTCATACCT	CCTCACGGCCTGGGATTT	(Korashy et al., 2016b)
rCYP1A1	CCAAACGAGTTCCGGCCT	TGCCCAAACCAAAGAGAATGA	(Korashy et al., 2016a)
rCYP1B1	GCTTTACTGTGCAAGGGAGACA	GGAAGGAGGATTCAAGTCAGGA	(Korashy et al., 2016a)
rCYCLIN D1	GCGAGCCATGCTTAAGACTGA	CTCCCTCTGCACGCACTTG	(Weroha et al., 2006)
rFOXO3a	CTATCTGGGCTGGGCAA	CTGGCTCAAGCACAACTTGG	(Alhoshani et al., 2020)
rBAX	CCCACCAGCTCTGAACAGTTC	GTGTCTCCCCAGCCATCCT	(Alhoshani et al., 2020)
rAPE1	CCCGCTCTAAGAATGTTGGTTG	CAGTGCTAGGTAAAGGGTGATGG	(Zhang et al., 2016)
rOGG1	GACTCAGACCGAGGATCAGCTC	GCTATAGAGCTGAGTCAGGCTGAC	(Zhang et al., 2010)
rβ-ACTIN	CCAGATCATGTTTGAGACCTTCAA	GTGGTACGACCAGAGGCATACA	(Korashy et al., 2016a)
hCYP1A1	CTATCTGGGCTGTGGGCAA	CTGGCTCAAGCACAACTTGG	(Maayah et al., 2015)
hβ-ACTIN	CCAGATCATGTTTGAGACCTTCAA	GTGGTACGACCAGAGGCATACA	(Alhoshani et al., 2020)

supplement and $1\times$ antibiotic/antimycotic, at 37 °C in humidified incubator containing 5% CO_2 as described before (Dontu et al., 2003; Wang et al., 2014). The mammosphere numbers and size were measured on Evos® inverted light microscope.

2.7. Statistical analysis

The significance of the statistical difference ($P < 0.05$) between control and treatment groups (mean ± SEM) were analyzed by One-Way Analysis of Variance followed by Student-Newman-Keul's test using Sigma Plot, Systat Software Inc, USA.

3. Results

3.1. Effect of MET on the expression of AhR and its regulated genes in response to DMBA in rat breast tissues

To examine the effect of MET on the expression of AhR, CYP1A1 and CYP1B1 in breast tissues isolated from rats treated with DMBA, we quantified the mRNA and protein expression of the target genes using RT-PCR and Western blot analyses, respectively. Treatment of rats with either DMBA (25 mg/kg) or TCDD (10 µg/kg) induced AhR mRNA levels by 6- and 12-fold ($P < 0.05$), respectively (Fig. 1A). The induction of AhR caused a proportional increase in the mRNA (Fig. 1B) and protein (Fig. 1C) levels of both CYP1A1 and CYP1B1. Importantly, pretreatment of rats with MET (500 mg/kg) significantly decreased the mRNA and protein expression levels of AhR, CYP1A1, and CYP1B1 to approximately their control levels.

3.2. Effect of MET on the apoptotic pathway in response to DMBA in rat breast tissues

Apoptosis is an important pathway that protects the cells from cancer initiation. To explore the protective effect of MET on cell apoptotic pathway, we measured the mRNA and protein expression levels of two pro-apoptotic markers, caspase-3 and BAX. Fig. 2A demonstrates that treatment of rats with DMBA and TCDD inhibited the cell apoptotic pathway by reducing the mRNA levels of caspase-3 and BAX by approximately 90% ($P < 0.05$), whereas significantly inhibited only BAX at the protein level (Fig. 2B). Importantly, MET 500 mg/kg pretreatment not only restored the inhibitory effect of DMBA but also induced apoptosis as evidenced by the upregulation of caspase-3 and BAX mRNA and protein levels (Fig. 2A and B).

FoxO3a is a driving force for apoptosis, however it is not clear yet whether MET-induced apoptosis is mediated through the modulation of FoxO3a. To examine this possibility, we investigated the effect of MET on FoxO3a expression. Fig. 2C shows that treatment with either DMBA or TCDD causes dramatic inhibition of FoxO3a

mRNA to <25%, whereas MET pretreatment restored the inhibition by DMBA and further induced FoxO3a mRNA level by 7-fold.

3.3. Effect of MET on the oxidative DNA damage and cell cycle deregulation in response to DMBA in rat breast tissues

DNA damage in response to chemical carcinogens is the first step toward cancer initiation. Thus, we tested the changes in the mRNA levels of base excision repair genes (APE1 and OGG1), markers of oxidative DNA damage, in response to DMBA and the protective effect of MET. Treatment with DMBA or TCDD significantly induced the mRNA levels of APE1 and OGG1 to about 2-fold by DMBA and more than 4-fold by TCDD, respectively (Fig. 3A). In the presence of MET, induction of APE1 was completely reversed to control levels, whereas no changes were observed on OGG1 level.

To further investigate the effect on cell cycle proliferation, we examined the expression of cyclin D1, a cell cycle proliferative gene, in response to DMBA and MET. Fig. 3B shows that treatment with DMBA or TCDD significantly increased the cell cycle proliferation by about 2- and 3-fold, respectively. However, pretreatment with MET caused cell cycle arrest as evidenced by complete inhibition (90%) of cyclin D1 mRNA levels.

3.4. Effect of MET on mammospheres formation in in vitro MCF-7 cells

In a previous study, we have shown DMBA-induced AhR activation enhanced CSCs development in MCF-7 cell line (Al-Dhfyan et al., 2017). Thus, in the present study we tested whether MET could block the DMBA-induced mammospheres formation in MCF-7 cells, a well-known marker for cancer stem cells. Fig. 4 demonstrates that induction of CYP1A1 mRNA levels in response to DMBA and TCDD was markedly induced by approximately 7- and 12-fold, respectively, which was accompanied with a proportional increase in the number of mammosphere formation by 1.5- and 2-fold, respectively. Interestingly, MET pretreatment significantly blocked the induction of CYP1A1 and mammospheres formation by DMBA to control levels.

4. Discussion

Carcinogenicity of AhR inducers indicates a role for the AhR/CYP1A1 pathway in cancer initiation, based on the fact that DMBA fails to induce cancer in cyp1 knockout mice (Shimada and Fujii-Kuriyama, 2004). Previous studies from our laboratory and others show a strong role for the activation of AhR/CYP1A1 pathways in breast carcinogenesis in several breast cancer cells (Al-Dhfyan et al., 2017; Do et al., 2014; Rodriguez and Potter, 2013; Maayah et al., 2015). Thus, attenuation of the AhR/CYP1A1 pathway could be a novel chemoprevention strategy to protect human tissues from the environmental carcinogenic compounds. Although sev-
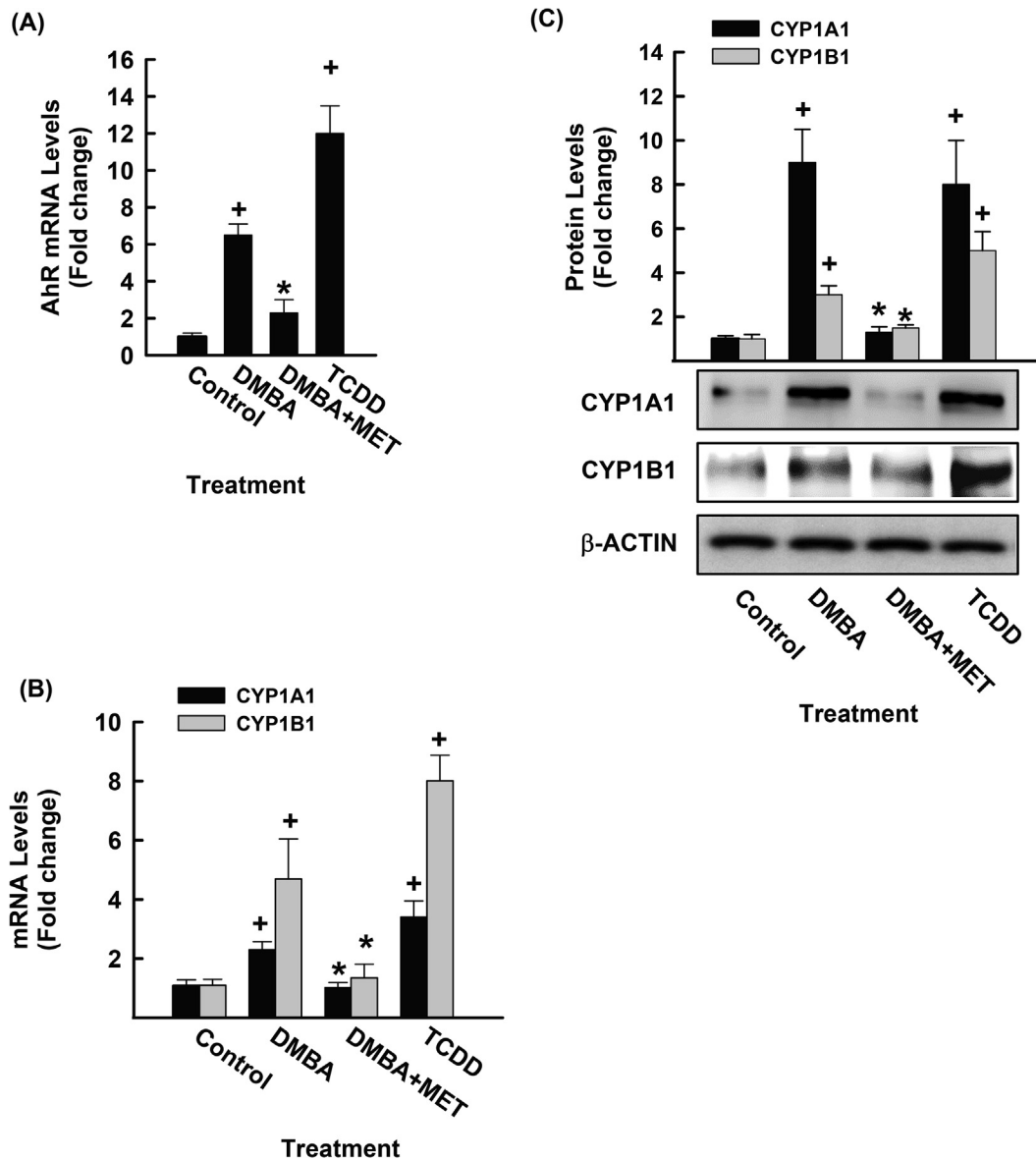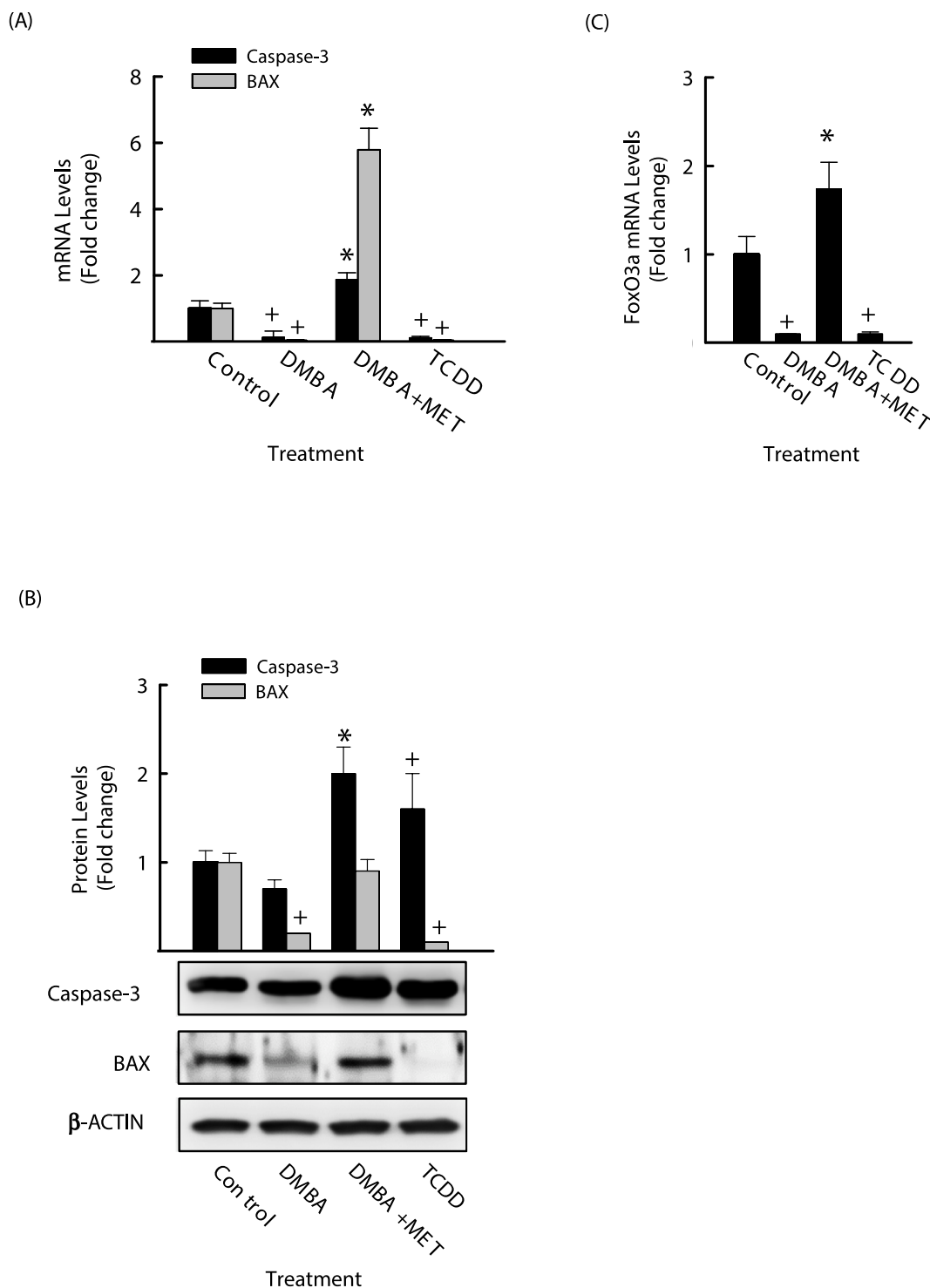
Fig. 1. **Effect of MET on AhR, CYP1A1 and CYP1B1 expressions in rat breast tissues**. WA rats were treated with TCDD (10 μg/kg) or DMBA (25 mg/kg) and in combination with MET (500 mg/kg). (A and B) The RT-PCR relative mRNA levels of AhR, CYP1A1 and CYP1B1 (mean ± SEM, n = 6 in triplicate). (C) The Western blot analysis of CYP1A1 and CYP1B1 proteins (mean ± SEM, n = 3 in triplicate). + P < 0.05 compared with control; *P < 0.05 compared with DMBA treatment.

eral chemopreventive agents are available and clinically used, identifying more safer drugs with less serious adverse effects are needed. Among these promising chemopreventive agents, MET is clinically used oral anti-hyperglycemic agent, relatively inexpensive with high safety margin and less side effects. Epidemiological studies on MET and cancer have shown an association between MET treatment and decrease the incidence of breast cancer in diabetic patients (Aljada and Mousa, 2012; Franciosi et al., 2013). Preclinical in vitro study has reported that MET can protect against breast carcinogenesis in MCF10A cells through AhR/CYP1A1-mediated mechanism (Maayah et al., 2015). However, the chemopreventive effects of MET against in vivo rat model of AhR-induced breast carcinogenesis were not investigated thoroughly. Therefore, to test the hypothesis that MET protects against AhR-induced animal breast carcinogenesis, Wister albino rats treated with DMBA in the presence and absence of MET were utilized as a study model. Similarly TCDD was also utilized as a positive control since it is the most potent and well-known AhR activator.

In the current study, induction of breast cancer initiation with DNA damage was achieved via treatment of the rats with single dose of DMBA or TCDD for only 4 weeks which resulted in a) activation of AhR and induction of CYP1A, well-known mediators of cancer initiation (Al-Dhfyan et al., 2017), b) downregulation of apoptotic markers caspase-3 and BAX and the tumor suppressor gene, FoxO3a, c) activation of oxidative DNA damage genes OGG1 and APE1 and cell cycle proliferative marker cyclin D1. However, the chemoprotective effects of MET against DMBA-induced rat breast carcinogenesis were evidenced by the ability of MET to restore DMBA effects through a) induction of pro-apoptosis, b) downregulation of cell cycle proliferation, and c) inhibition of oxidative DNA damage. However, these effects were more pronounced by TCDD than DMBA.

Apoptosis is one of the mechanisms by which the cell protects itself from cancer initiation and DNA adduct formation. Thus, apoptotic markers are targeted by carcinogens as well as by anticancer agents. In this regard, we reported here that MET induced

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Fig. 2. **Effect of MET on caspase-3, BAX, and FoxO3a expressions in rat breast tissues.** WA rats were treated with TCDD (10 µg/kg) or DMBA (25 mg/kg) and in combination with MET (500 mg/kg). (A and C) The RT-PCR relative mRNA levels of Caspase-3, BAX and FoxO3a (mean ± SEM, n = 6 in triplicate). (B) The Western blot analysis of Caspase-3 and BAX protein (mean ± SEM, n = 3 in triplicate). + P < 0.05 compared with control; *P < 0.05 compared with DMBA treatment.

cell apoptosis through inhibition of the AhR/CYP1A1 activation and induction of FoxO3a. This is supported by the connection between AhR/CYP1A1 activation and apoptosis (Huang et al., 2018) and the ability of MET to decrease the induction of AhR pathway. The link between MET inhibitory effect on the AhR/CYP1A1 pathway in breast cancer cell lines (Do et al., 2014; Maayah et al., 2015) and AhR-mediated apoptosis suggest the possible pro-apoptotic property of MET (Gao et al., 2016; Ma et al., 2019). In addition, induc-

tion of Foxo3a gene, which act as a tumor suppressor in cancer, is another postulated mechanism of induction of apoptosis by MET. This is also supported by first; overexpression of FoxO3a inhibits cancer initiation and progression (Liu et al., 2018) and second; several chemotherapeutic agents exhibit their effects through activating FoxO3a pathway (Korashy et al., 2016b; Sun et al., 2018). Although the ability of MET to induce FoxO3a was reported before in in vitro human breast cancer cells (Queiroz et al., 2014), the cur-
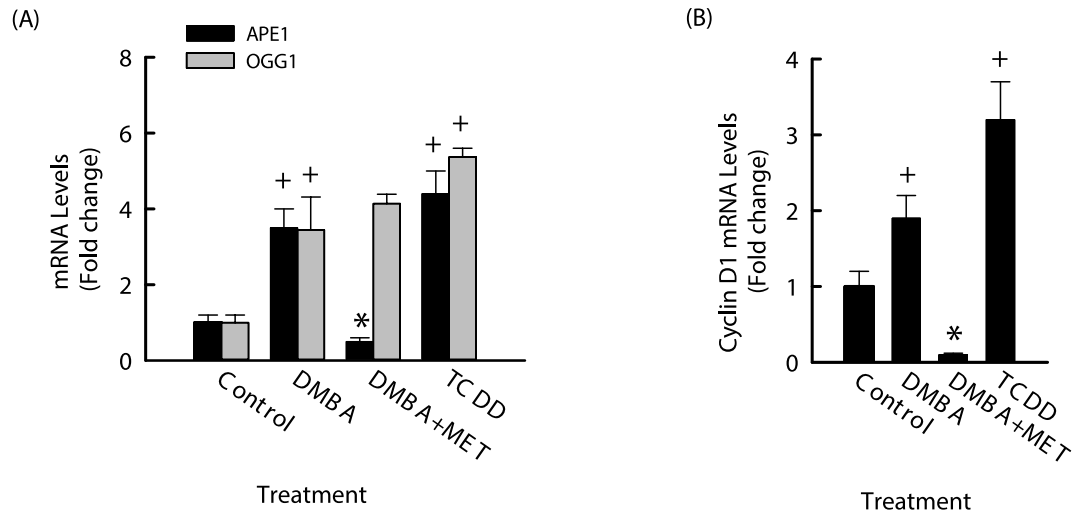
Fig. 3. **Effect of MET on APE1, OGG1, and Cyclin D1 expressions in rat breast tissues.** WA rats were treated with TCDD (10 μg/kg) or DMBA (25 mg/kg) and in combination with MET (500 mg/kg). The relative mRNA levels of APPE OGG1 (A) and cyclin D1 (B) were quantified by RT-PCR, (mean ± SEM, n = 6 in triplicate). + P < 0.05 compared with control; *P < 0.05 compared with DMBA treatment.
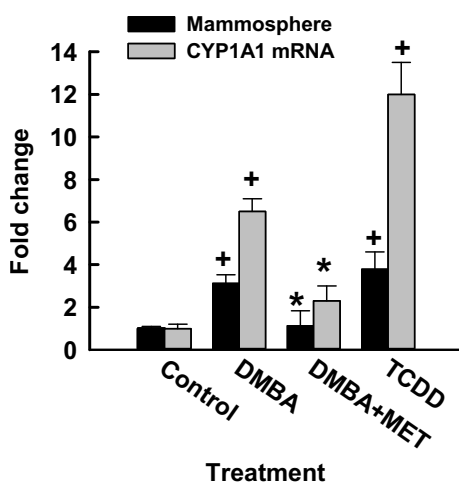
Fig. 4. **Effects of MET on CYP1A1 gene expression and mammospheres formation in MCF-7 cells.** MCF-7 cells were treated with TCDD (10 nM) or DMBA (5 μM) for 72 h in the presence and absence of MET (4 mM). The spheroid cell numbers and size (mammospheres) and the CYP1A1 mRNA levels were determined, (Mean ± SEM, n = 6 in triplicate), +P < 0.05 compared with control; *P < 0.05 compared with DMBA treatment.

rent study is the first to report the induction of FoxO3a gene by MET in in vivo rat breast tissues.

FoxO3a is a cellular mediator of cancer cells in response to oxidative stress by converging oxidative stress signaling to cell cycle arrest and DNA damage. Alteration of the expression and activity of DNA repair genes and cell cycle proliferative markers are considered as markers for cancer initiation (Broustas and Lieberman, 2014). Previous in vitro studies showed that DMBA-induced cancer initiation and adduct formation is associated with induction of cell proliferative and DNA repair genes (Braithwaite et al., 1998; Sahin et al., 2011). The hypotheses that MET slow-downs cell proliferation and reduces DNA oxidative damage have been proved in this study, in which MET treatment specifically inhibited the oxidative DNA damage gene, APE1, and induced cell cycle arrest through downregulation of cyclin D1 (Gwak et al., 2017; Zhuang and Miskimins, 2008). Our in vivo results are supported by previous studies showed that MET inhibited APE1 DNA

repair genes in human breast epithelia MCF10A cells treated with DMBA (Maayah et al., 2015). The inhibitory effect of MET on cell cycle genes has been reported in several in vitro models of breast (Zhuang and Miskimins, 2008), ovarian (Gwak et al., 2017), and prostate (Ben Sahra et al., 2008) cancer, however, to the best of our knowledge, this is first to report the cell cycle arrest effect of MET in in vivo rat model of breast cancer.

Activation of AhR/CYP1A1 pathway mediates cancer initiation through enhancing the proliferation and self-renewal of breast CSCs (Al-Dhfyan et al., 2017; Stanford et al., 2016). In this context, we showed that knockdown of AhR or CYP1A1 resulted in inhibition of breast CSCs formation (Al-Dhfyan et al., 2017). These studies and the inhibitory effect of MET on AhR/CYP1A1 raised the question of whether MET is able to reduce breast CSCs formation. We addressed this question using in vitro human breast cancer MCF-7 cells model, where MET treatment significantly reduced DMBA-induced mammospheres formation, a well-known breast CSCs marker, which was accompanied by a proportional decrease in CYP1A1 expression. Although the effect of MET on mammospheres formation was reported before (Hirsch et al., 2009), this is the first to report a link between MET effect on mammospheres formation and CYP1A1 expression. The molecular mechanisms through which MET inhibits AhR/CYP1A1 pathway was comprehensively explored by our group (Maayah et al., 2015). In which, we have shown that MET inhibits DMBA-induced XRE luciferase reporter gene expression in murine hepatoma Hepa1c1c7 cells, that is only mediated through AhR, through a transcriptional mechanism. However, using Competitive ligand binding assay in guinea pig cytosol, MET was not able to directly bind to AhR suggesting a ligand-independent inhibitory mechanism (Maayah et al., 2015). This could be postulated that MET could bind to a second binding site on the AhR rather than DMBA binding site.

In conclusion, the present study provides the mechanistic evidence that MET exhibits chemopreventive activity in vivo rat model of breast carcinogenesis through inhibition of the AhR/CYP1A1-induced breast carcinogenesis and inhibition of CSCs formation. However, the main limitation of the study is that most of the work was conducted at the mRNA and protein levels only, whereas no activities studies were performed. Thus, studies measuring the enzymatic activities of the target proteins are required to further confirm the results obtained in the study.

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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References

Abrams, T.J., Lee, L.B., Murray, L.J., Pryer, N.K., Cherrington, J.M., 2003. SU11248 inhibits KIT and platelet-derived growth factor receptor beta in preclinical models of human small cell lung cancer. Mol Cancer Ther 2, 471–478.

Al-Dhfyan, A., Alhoshani, A., Korashy, H.M., 2017. Aryl hydrocarbon receptor/cytochrome P450 1A1 pathway mediates breast cancer stem cells expansion through PTEN inhibition and beta-Catenin and Akt activation. Mol Cancer 16, 14.

Alhoshani, A., Alanazi, F.E., Alotaibi, M.R., Attwa, M.W., Kadi, A.A., Aldhfyan, A., Akhtar, S., Hourani, S., Agouni, A., Zeidan, A., Korashy, H.M., 2020. EGFR Inhibitor Gefitinib Induces Cardiotoxicity through the Modulation of Cardiac PTEN/Akt/FoxO3a Pathway and Reactive Metabolites Formation. In Vivo and in Vitro Rat Studies. Chem Res Toxicol 33, 1719–1728.

Alhaider, A.A., Korashy, H.M., Sayed-Ahmed, M.M., Mobark, M., Kfoury, H., Mansour, M.A., 2011. Metformin attenuates streptozotocin-induced diabetic nephropathy in rats through modulation of oxidative stress genes expression. Chem Biol Interact 192, 233–242.

Aljada, A., Mousa, S.A., 2012. Metformin and neoplasia: implications and indications. Pharmacol Ther 133, 108–115.

Bekki, K., Vogel, H., Li, W., Ito, T., Sweeney, C., Haarmann-Stemmann, T., Matsumura, F., Vogel, C.F., 2015. The aryl hydrocarbon receptor (AhR) mediates resistance to apoptosis induced in breast cancer cells. Pestic. Biochem. Physiol. 120, 5–13.

Ben Sahra, I., Laurent, K., Loubat, A., Giorgetti-Peraldi, S., Colosetti, P., Auberger, P., Tanti, J.F., Le Marchand-Brustel, Y., Bost, F., 2008. The antidiabetic drug metformin exerts an antitumoral effect in vitro and in vivo through a decrease of cyclin D1 level. Oncogene 27, 3576–3586.

Bowker, S.L., Majumdar, S.R., Veugelers, P., Johnson, J.A., 2006. Increased cancer-related mortality for patients with type 2 diabetes who use sulfonylureas or insulin. Diabetes Care 29, 254–258.

Braithwaite, E., Wu, X., Wang, Z., 1998. Repair of DNA lesions induced by polycyclic aromatic hydrocarbons in human cell-free extracts: involvement of two excision repair mechanisms in vitro. Carcinogenesis 19, 1239–1246.

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R.L., Torre, L.A., Jemal, A., 2018. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J. Clin. 68, 394–424.

Broustas, C.G., Lieberman, H.B., 2014. DNA damage response genes and the development of cancer metastasis. Radiat Res 181, 111–130.

Buters, J.T., Sakai, S., Richter, T., Pineau, T., Alexander, D.L., Savas, U., Doehmer, J., Ward, J.M., Jefcoate, C.R., Gonzalez, F.J., 1999. Cytochrome P450 CYP1B1 determines susceptibility to 7, 12-dimethylbenz[a]anthracene-induced lymphomas. Proc Natl Acad Sci U S A 96, 1977–1982.

Chidambaram, N., Baradarajan, A., 1996. Influence of selenium on glutathione and some associated enzymes in rats with mammary tumor induced by 7,12-dimethylbenz(a)anthracene. Mol Cell Biochem 156, 101–107.

Do, M.T., Kim, H.G., Tran, T.T., Khanal, T., Choi, J.H., Chung, Y.C., Jeong, T.C., Jeong, H.G., 2014. Metformin suppresses CYP1A1 and CYP1B1 expression in breast cancer cells by down-regulating aryl hydrocarbon receptor expression. Toxicol Appl Pharmacol 280, 138–148.

Dontu, G., Abdallah, W.M., Foley, J.M., Jackson, K.W., Clarke, M.F., Kawamura, M.J., Wicha, M.S., 2003. In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes Dev 17, 1253–1270.

Evans, J.M., Donnelly, L.A., Emslie-Smith, A.M., Alessi, D.R., Morris, A.D., 2005. Metformin and reduced risk of cancer in diabetic patients. BMJ 330, 1304–1305.

Falah, R.R., Talib, W.H., Shbailat, S.J., 2017. Combination of metformin and curcumin targets breast cancer in mice by angiogenesis inhibition, immune system modulation and induction of p53 independent apoptosis. Ther Adv Med Oncol 9, 235–252.

Ferreira, I., Ferreira, J., Vollet-Filho, J.D., Moriyama, L.T., Bagnato, V.S., Salvadori, D.M., Rocha, N.S., 2013. Photodynamic therapy for the treatment of induced mammary tumor in rats. Lasers Med Sci 28, 571–577.

Franciosi, M., Lucisano, G., Lapice, E., Strippoli, G.F., Pellegrini, F., Nicolucci, A., 2013. Metformin therapy and risk of cancer in patients with type 2 diabetes: systematic review. PLoS ONE 8, e71583.

Gao, Z.Y., Liu, Z., Bi, M.H., Zhang, J.J., Han, Z.Q., Han, X., Wang, H.Y., Sun, G.P., Liu, H., 2016. Metformin induces apoptosis via a mitochondria-mediated pathway in human breast cancer cells in vitro. Exp Ther Med 11, 1700–1706.

Gwak, H., Kim, Y., An, H., Dhanasekaran, D.N., Song, Y.S., 2017. Metformin induces degradation of cyclin D1 via AMPK/GSK3beta axis in ovarian cancer. Mol Carcinog 56, 349–358.

Hirsch, H.A., Iliopoulos, D., Tsichlis, P.N., Struhl, K., 2009. Metformin selectively targets cancer stem cells, and acts together with chemotherapy to block tumor growth and prolong remission. Cancer Res 69, 7507–7511.

Huang, Y., He, J., Liang, H., Hu, K., Jiang, S., Yang, L., Mei, S., Zhu, X., Yu, J., Kijlstra, A., et al., 2018. Aryl Hydrocarbon Receptor Regulates Apoptosis and Inflammation in a Murine Model of Experimental Autoimmune Uveitis. Front Immunol 9, 1713.

Ip, C., 1996. Mammary tumorigenesis and chemoprevention studies in carcinogen-treated rats. J Mammary Gland Biol Neoplasia 1, 37–47.

Jung, J.W., Park, S.B., Lee, S.J., Seo, M.S., Trosko, J.E., Kang, K.S., 2011. Metformin represses self-renewal of the human breast carcinoma stem cells via inhibition of estrogen receptor-mediated OCT4 expression. PLoS ONE 6, e28068.

Kerzee, J.K., Ramos, K.S., 2001. Constitutive and inducible expression of Cyp1a1 and Cyp1b1 in vascular smooth muscle cells: role of the Ahr bHLH/PAS transcription factor. Circ Res 89, 573–582.

Korashy, H.M., Attafi, I.M., Ansari, M.A., Assiri, M.A., Belali, O.M., Ahmad, S.F., Al-Alallah, I.A., Anazi, F.E., Alhaider, A.A., 2016a. Molecular mechanisms of cardiotoxicity of gefitinib in vivo and in vitro rat cardiomyocyte: Role of apoptosis and oxidative stress. Toxicol. Lett. 252, 50–61.

Korashy, H.M., Belali, O.M., Ansar, M.A., Alharbi, N.O., 2016b. FoxO3a is Essential for the Antiproliferative and Apoptogenic Effects of Sunitinib in MDA-MB231 Cell Line. Anticancer Res 36, 6097–6108.

Liu, Y., Ao, X., Ding, W., Ponnusamy, M., Wu, W., Hao, X., Yu, W., Wang, Y., Li, P., Wang, J., 2018. Critical role of FOXO3a in carcinogenesis. Mol Cancer 17, 104.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402–408.

Ma, L., Wei, J., Wan, J., Wang, W., Wang, L., Yuan, Y., Yang, Z., Liu, X., Ming, L., 2019. Low glucose and metformin-induced apoptosis of human ovarian cancer cells is connected to ASK1 via mitochondrial and endoplasmic reticulum stress-associated pathways. J Exp Clin Cancer Res 38, 77.

Maayah, Z.H., Ghebeh, H., Alhaider, A.A., El-Kadi, A.O., Soshilov, A.A., Denison, M.S., Ansari, M.A., Korashy, H.M., 2015. Metformin inhibits 7,12-dimethylbenz[a]anthracene-induced breast carcinogenesis and adduct formation in human breast cells by inhibiting the cytochrome P4501A1/aryl hydrocarbon receptor signaling pathway. Toxicol. Appl. Pharmacol. 284, 217–226.

NIH (2011). In Guide for the Care and Use of Laboratory Animals, th, ed. (Washington (DC)).

Papadakis, M.A., McPhee, S.J., Bernstein, J., 2021. Breast Cancer, Female. Quick Medical Diagnosis & Treatment 2021. McGraw-Hill Education, New York, NY.

Pollenz, R.S., 2002. The mechanism of AH receptor protein down-regulation (degradation) and its impact on AH receptor-mediated gene regulation. Chem Biol Interact 141, 41–61.

Queiroz, E.A., Puukila, S., Eichler, R., Sampaio, S.C., Forsyth, H.L., Lees, S.J., Barbosa, A.M., Dekker, R.F., Fortes, Z.B., Khaper, N., 2014. Metformin induces apoptosis and cell cycle arrest mediated by oxidative stress, AMPK and FOXO3a in MCF-7 breast cancer cells. PLoS ONE 9, e98207.

Rodriguez, M., Potter, D.A., 2013. CYP1A1 regulates breast cancer proliferation and survival. Mol Cancer Res 11, 780–792.

Rueden, C.T., Schindelin, J., Hiner, M.C., DeZonia, B.E., Walter, A.E., Arena, E.T., Eliceiri, K.W., 2017. Image J2: ImageJ for the next generation of scientific image data. BMC Bioinf. 18, 529.

Sahin, K., Tuzcu, M., Sahin, N., Akdemir, F., Ozercan, I., Bayraktar, S., Kucuk, O., 2011. Inhibitory effects of combination of lycopene and genistein on 7,12- dimethyl benz(a)anthracene-induced breast cancer in rats. Nutr Cancer 63, 1279–1286.

Shimada, T., Fujii-Kuriyama, Y., 2004. Metabolic activation of polycyclic aromatic hydrocarbons to carcinogens by cytochromes P450 1A1 and 1B1. Cancer Sci 95, 1–6.

Stanford, E.A., Wang, Z., Novikov, O., Mulas, F., Landesman-Bollag, E., Monti, S., Smith, B.W., Seldin, D.C., Murphy, G.J., Sherr, D.H., 2016. The role of the aryl hydrocarbon receptor in the development of cells with the molecular and functional characteristics of cancer stem-like cells. BMC Biol 14, 20.

Sun, L., Huang, Y., Liu, Y., Zhao, Y., He, X., Zhang, L., Wang, F., Zhang, Y., 2018. Ipatasertib, a novel Akt inhibitor, induces transcription factor FoxO3a and NF-kappaB directly regulates PUMA-dependent apoptosis. Cell Death Dis 9, 911.

Thompson, M.D., Grubbs, C.J., Bode, A.M., Reid, J.M., McGovern, R., Bernard, P.S., Stijleman, I.J., Green, J.E., Bennett, C., Juliana, M.M., et al., 2015. Lack of effect of metformin on mammary carcinogenesis in nondiabetic rat and mouse models. Cancer Prev Res (Phila) 8, 231–239.

Weroha, S.J., Li, S.A., Tawfik, O., Li, J.J., 2006. Overexpression of cyclins D1 and D3 during estrogen-induced breast oncogenesis in female ACI rats. Carcinogenesis 27, 491–498.

Wang, R., Lv, Q., Meng, W., Tan, Q., Zhang, S., Mo, X., Yang, X., 2014. Comparison of mammosphere formation from breast cancer cell lines and primary breast tumors. J Thorac Dis 6, 829–837.

White, A.J., Bradshaw, P.T., Hamra, G.B., 2018. Air pollution and Breast Cancer: A Review. Curr Epidemiol Rep 5, 92–100.

Whitelaw, M.L., Gottlicher, M., Gustafsson, J.A., Poellinger, L., 1993. Definition of a novel ligand binding domain of a nuclear bHLH receptor: co-localization of ligand and hsp90 binding activities within the regulable inactivation domain of the dioxin receptor. Embo J 12, 4169–4179.

Whitlock Jr., J.P., 1999. Induction of cytochrome P4501A1. Annu Rev Pharmacol Toxicol 39, 103–125.

Zhang, X., Xu, Z., Lin, F., Wang, F., Ye, D., Huang, Y., 2016. Increased Oxidative DNA Damage in Placenta Contributes to Cadmium-Induced Preeclamptic Conditions in Rat. Biol Trace Elem Res 170, 119–127.

Zhang, Y., Zhang, L., Zhang, L., Bai, J., Ge, H., Liu, P., 2010. Expression changes in DNA repair enzymes and mitochondrial DNA damage in aging rat lens. Mol Vis 16, 1754–1763.

Zhuang, Y., Miskimins, W.K., 2008. Cell cycle arrest in Metformin treated breast cancer cells involves activation of AMPK, downregulation of cyclin D1, and requires p27Kip1 or p21Cip1. J Mol Signal 3, 18.