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Abstract: The carcinogenic capacity of Bisphenol A (BPA) at nano-molar concentrations of 8.73 and 17.47 nM (in culture) was evaluated on both normal breast epithelial cells (MCF-10A) and breast cancer cells (MCF-7). The highest DNA damage was recorded at 6 h and MCF-10A cells showed significant increase of IGF1R protein while mRNA expression was unchanged; however, the converse was true for MCF-7 cells. Homology modeling predicted the structure of SPCA1/2 and indicated BPA binding within catalytic domain. Our data indicated that BPA caused detectable DNA damage, inhibited cellular SPCA1/2 protein which eventually dysregulated Ca\textsuperscript{2+}-dependent IGF1R.

Subjects: Bioscience; Pharmacology; Cancer; Health and Social Care; Cancer

Keywords: BPA; DNA damage; breast cancer; tumorigenesis; cell signaling

1. Introduction
Being a synthetic carbon compound bisphenol A (BPA) is considered an endocrine disruptor (US Food and Drug Administration, 2008). BPA related toxicity in human body is still a controversy (EFSA Panel on Food Contact Materials, 2015; Health Council of the Netherlands, 2019). Previous toxicological studies report, a high dose of BPA (3400 mg/kg of body weight) as toxic. At this high concentration unspecified changes in the gastrointestinal tract, liver, kidney, ureter and bladder

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PUBLIC INTEREST STATEMENT

Bisphenol A (BPA) is one of the most abundant organic ingredients in plastics and epoxy resins. Bearing structural resemblance to the female reproductive hormone estrogen, its therapeutic use was discontinued in the late 1950s due to its cancer-causing potential. This study provides a biological link between low-dose exposure to BPA and identifies three cellular steps that can increase the risk of cancer as studied in cultured normal breast epithelial cell line MCF-10A. In step 1, a nano-molar concentration of BPA can potentiate DNA damage. Step 2 is characterized by cytochrome P450Z1 (CYP4Z1) over expression, while in step 3, we report that a calcium trans- 

porter called secretory pathway calcium ATPase1/2 (SPCA1/2) is inhibited. This inhibition possibly causes misfolding of the calcium requiring insulin-like growth factor 1 receptor (IGF1R) or its anterograde trafficking dysregulation. Both CYP4Z1 and IGF1R are markers for breast cancer.
occur in rats (Yamasaki et al., 2003). Microgram quantities of BPA can leach from the plastic container into the contents (Brede, Fjeldal, Skjervak, & Herikstad, 2003), while the median concentration of BPA leached from plastic bottles is 0.1 ng/mL (Dhaini & Nassif, 2014). We previously argued that low-dose BPA can cause DNA damage and activate downstream signaling that could potentially disrupt insulin growth factor 1 (IGF1R) surface receptor (Jalal, Surendranath, Pathak, Yu, & Chung, 2017). The objective of this project was to understand the various cellular tumorigenic events, including DNA damage and early tumor markers such as IGF1R and CYP4Z1 protein.

The BPA analogue bisphenol AF (BPAF), besides BPA itself, induces neurotoxic effects on hippocampal cell line (HT-22) via increased intracellular Ca\(^{2+}\) leading to oxidative stress through MAPK pathway (Bermudez, Gray, & Wilson, 2010; Tewar et al., 2016). However, the BPA-induced neurotoxic (Rebuli et al., 2015, 2014), reproductive (Delclos et al., 2016; Wang et al., 2016) and DNA damaging role in ovaries have already been widely studied but the temporal or low-dose kinetics involved remain elusive (Ganesan & Keating, 2016). The formation of DNA adducts in-vitro is concentration-dependent within a range of 6.2–100 μM, corresponding to doses of 14.25–228 ng (Izzotti, Kanitz, D’Agostini, Camoirano, & De Flora, 2009). BPA is metabolized to its glucuronide and hydroxylated derivatives such as 3-hydroxy-BPA (3-OH-BPA or BPA catechol), which is further oxidized to BPA-3, 4-quinone that forms a guanine-N7 adduct (deoxyguanosine) to cause direct DNA damage (Edmonds et al., 2004). The involvement of NF-κB and its migration into the nucleus indicates inflammation pathway activation (Zhu et al., 2015). A BPAF-induced and MAPK mediated apoptosis confirms the involvement of MAPK pathway (Lee, Kim, Shin, & Kim, 2013). An in-vivo maternal exposure to BPA during lactation can increase the risk of mammary carcinogenesis in rodents. BPA-induced changes in PR-A, SRC 1, erbB3, and Akt activity increase cell proliferation and decrease apoptosis via the Akt and MAPK pathways which indicates elevated mammary cancer susceptibility (Jenkins et al., 2009). Through a long non-coding RNA (HOTAIR) BPA plays a central part in silencing genes while being transcriptionally regulated by estradiol in MCF-7 cells thus contributing to tumorigenic event progression. Estrogen response elements of HOTAIR in-vitro and in-vivo are induced by BPA and diethylstilbestrol (Bhan et al., 2014).

Being a second messenger, imbalance in the cytoplasmic Ca\(^{2+}\) sequestration triggers critical events that lead to tumorigenesis via apoptosis, cell motility or proliferation. Cytoplasmic Ca\(^{2+}\) is partly homeostasized via the secretory pathway of calcium ATPase which has two isoforms SPCA1 and SPCA2. SPCA1 occurs ubiquitously in vertebrates, including Humans, and has orthologs in lower eukaryotes (Missiaen, Dode, Vanoevelen, Raeymaekers, & Wuytack, 2007). SPCA1 (ATP2C1) mutations cause Hailey–Hailey disease that produces stratified epithelium of skin due to loss of intercellular adhesion or Acantholysis (Mascia, Denning, Kopen, & Yuspa, 2012; Missiaen et al., 2004). Both protein isoforms are ATP powered and pump Ca\(^{2+}\) and Mn\(^{2+}\) from cytosol into the lumen of Golgi for protein sorting, processing and glycosylation (Dürr et al., 1998). The siRNA gene silencing of SPCA1 has varying effects on the regulation of calcium-dependent enzymes in MDA MB231 cell line and SPCA1 knockdown in mice causes embryonic lethality (Okunade et al., 2007). SPCA1 inhibition in-vitro alters the cell surface levels of exogenously expressed proteins indicating a functional involvement of SPCA1 in cellular protein trafficking (Lissandron, Podini, Pizzo, & Pozzan, 2010). SPCA1 is a thapsigargin-sensitive intracellular Ca\(^{2+}\) pump found mostly in the Golgi compartment (Sagara, Fernandez-Belda, de Meis, & Inesi, 1992). Contribution of Ca\(^{2+}\) pump to cytosolic Ca\(^{2+}\) signaling in HeLa cells via RNA-mediated interference disrupts the baseline Ca\(^{2+}\) but detectable in the cytoplasm (Van Boelen et al., 2003). Moreover, Bis (2-hydroxy-3-tert-butyl-5-methyl-phenyl)-methane is a potent and selective inhibitor of SPCA1 (Lai & Michelangeli, 2012).

Inhibition of SPCA1 produces a significant alteration in the processing of insulin-like growth factor receptor 1 (IGF1R) with reduced levels of IGF1Rβ and accumulation of the inactive trans-Golgi network pro-IGF1R form (Grice et al., 2010). IGF1R is a pro-protein convertase substrate essential for mammary gland development and milk production (Yee & Wood, 2008). Expression of IGF1R is upregulated in breast cancer and has been associated with cancer initiation, evasion of apoptosis, motility, proliferation and resistance to cancer therapy (Aaltonen et al., 2014; Frasca
et al., 2008; Haupt, Ro, & Schwartz, 2010; Klinakis et al., 2009; Maor et al., 2007). Our previous study demonstrated the appearance of cytochrome P450Z1 (CYP4Z1) autoantibodies in the sera of breast cancer patients (Nunna, Jalal, & Bureik, 2017), while CYP4Z1 has a significantly higher expression in cancerous breast tissue (Rieger et al., 2004), indicating CYP4Z1 as an early diagnostic marker of breast cancer initiation. Hence IGF1R and CYP4Z1 can be used as early markers of tumorigenic initiation event. The broad spectrum effects of BPA on DNA damage, SPCA1/2, IGF1R and CYP4Z1 that lead to tumorigenesis are therefore crucial to our understanding of tumor initiation and progression.

2. Materials and methods

2.1. Cell culture
Breast epithelial carcinoma cells (MCF-7) were kindly provided by the institute of molecular biology, Nankai University, Tianjin, China, while the control breast epithelial cell line MCF-10A was purchased from the cell culture bank (Cat # C389 Stem Cell Bank, Chinese Academy of Sciences, Shanghai, China). BV2, MDA-MB-231 cell lines were already present in our lab. All cell lines were grown according to the ATCC guidelines.

2.2. BPA treatment
BPA (Xiya Reagents, Cas# 80–05–7) was used to prepare a stock of 1.25 g/250 mL in DMSO and serial dilutions carried out to prepare 2.0 and 4.0 µg/mL concentrations and 1 µL/mL dose used for treatment which translate into working concentrations of 8.73 and 17.47 nM, respectively. The desired drug treatment was applied to the cell culture for 3, 6, 24 or 48 h to quantify temporal effects. At the end of the designated time duration the medium was aspirated and cells washed with 1 X PBS before adding fresh medium into the cell culture. Pure sterile DMSO was used as internal control (labeled as mock in figures) in all experiments.

2.3. Alkaline comet assay to measure single-strand breaks (SSBs)
Alkaline comet assay described by (Collins, Dusinská, Gedik, & Stětina, 1996; Yamauchi, Kawai, & Ueda, 2002) was used to quantify SSBs while neutral comet assay was performed to measure DSBs in the target cell lines. The cells, following BPA treatment, were exposed to a 1-h treatment of bromodeoxyuridine (5-BrdU) to allow DNA labeling. After which time the cells were harvested by trypsinization for 5 min at 37°C and spun for 5 min at 1000 rpm to be resuspended in fresh 1X PBS at a density of 5 × 10^4 to 1 × 10^5 cells/mL and spun again at 1000 rpm. The cell pellet was then resuspended in 100 µL of low-melting agarose at 37°C and added 30 µL of this suspension in the center of a glass slide covered with a layer of regular agarose having a hole punched in the center. Lysis and neutralization treatment was followed as outlined (Collins et al., 1996, 2001; Yamauchi et al., 2002).

2.4. Neutral comet assay to measure double-strand breaks (DSBs)
We modified the protocol presented (Boutet-Robinet, Trouche, & Canitrot, 2013) for bromodeoxyuridine (BrdU) labeling of DNA. Following BPA treatment, the cells were exposed to a 1-h treatment of BrdU to allow DNA labeling. For rest of the lysis and neutralization treatment, we followed the protocol cited above.

2.5. Quantification of DNA damage
BPA-induced DNA damage was measured in all four target cell lines. A 24-well plate was used to culture cells in complete growth medium for 48 h in 5% CO_2. Once attached, the cells were treated with doses of BPA as mentioned before. The temporal and dose effects were measured using alkaline comet assay. Cells were lysed and electrophoresed after embedding in low melting agarose on glass slides. BrdU-labeled DNA breaks were visualized by fluorescently tagged secondary antibody against the primary anti-BrdU. Microscopic images were analyzed for tail length (using IMAGINE software accessible from http://faculty.pieas.edu.pk/fayyaz/software.html#imagine) and 3D plots of the whole cell used for measuring Z-ratio of electrophoresed DNA using
IMAGE J software (data not shown). The tail length of the comets was measured in arbitrary units however presented in pixels for easy comprehension of reader. The data was normalized and average tail lengths of all experimental time points from comets were measured and plotted to observe the temporal effect.

2.6. Antibody tagging for comet assay, Western analysis and IHC

Anti-BrdU mouse monoclonal antibody (Biolegend cat #339801) was diluted 1:250 in dilution buffer prepared in bovine serum albumin (BSA). Secondary antibody for comet assay was donkey anti-mouse Alexa 488 conjugate (Santa Cruz sc-362248) used at 1:5000 dilution. We used NF-κB sampler kit provided by Cell Signaling (cat # 9936) for primary antibodies. The NF-κB p65/RelA Rabbit monoclonal antibody (cat # 8242), phospho NF-κB (cat # 3033) rabbit monoclonal, IκB-α rabbit monoclonal (cat# 4814) and phospho IκB-α rabbit monoclonal (cat# 2859) were used with concentration of 1:1000 (in BSA with 0.1% sodium azide) for western analysis (data not shown). The primary antibody for SPCA1/2 (Santa Cruz cat # 134450) was a rabbit polyclonal IgG used at a concentration of 1:1000 in BSA dilution buffer. The PVDF membrane (after blocking with 5% w/v skimmed milk in TBST) was incubated in 10 mL of primary antibody overnight with gentle shaking at 4°C. Secondary antibody for SPCA1/2 was goat anti-rabbit IgG (H&L) with HRP (Affinity Cat # S0001) used at 1:2000. The primary antibody for IGF1R (Abcam Cat # 39675) was a rabbit polyclonal IgG used with concentration of 1:1000 in BSA dilution buffer. Secondary antibody for IGF1R was goat anti-rabbit IgG, HRP linked antibody (SC cat# 7074) at a concentration of 1:2000. To detect primary antibody the PVDF membrane was incubated for 1 h at room temperature with secondary antibody (diluted in 10 mL of blocking buffer). Loading control was β-Actin and detected using (SC cat# 47778) mouse monoclonal at 1:1000, probed with secondary goat anti mouse (H&L) HRP (Affinity cat# S0002) used at 1:2000.

2.7. Microscopy

All microscopic analyses were done on Nikon ECLIPSE 80i and used UV filter for detecting DAPI labeled nuclei, FITC for detecting anti-BrdU-labeled DNA breaks. Analyses were done at a magnification of X40 for comet assay, and at X100 (using oil immersion) for IHC (see below for details) and up to 10 random images captured for analysis.

2.8. Comet score

Image J software was used for surface plots to measure the length of comet tail as a measure of extent of DNA damage, while Imagine software was used for counting the DNA breaks labeled by Alexa 488 conjugated secondary antibody against anti-BrdU to selectively count the green spots. Both quantifications were normalized against mock and plotted to show the effect of BPA treatment.

2.9. Western blot

The western blot was performed as described previously by Zhu et al. (2015). Briefly, the confluent cells (80%) were lysed with fresh lysis buffer containing 1x protease inhibitor, 1x phosphatase inhibitor, 20% Triton X-100 in 1X PBS. The protein was quantified using BCA assay (Smith et al., 1985). After electrophoresis the proteins were transferred to a 0.2-μm PVDF membrane at 12 V for 1.5 h. After blocking the membrane in 5% milk/TBST, it was incubated in primary antibody (1:1000) overnight at 4°C with gentle shaking, or 3 h at room temperature. Excess antibody was washed off with gentle shaking in 1X TBST three times (5 min each). The membrane was incubated with secondary antibody HRP-conjugated IgG (diluted 1:2500) for 1 h at room temperature and excess antibody again washed off with three TBST washes. The protein of interest was detected with ECL (Western Bright ECL, Advansta Inc., K-12045-D50). Band intensities were quantified with Image J software by subtracting the background intensities.

2.10. Immunohistochemistry (IHC)

The immunohistochemistry was performed as mentioned earlier (Nunna et al., 2017). Briefly, confluent MCF-7 and MCF-10A cells cultured on flamed cover slips were washed with ice cold PBS (1X) and then fixed for 5 min using 4% paraformaldehyde. After blocking with 5% milk in TBST,
a 1:1000 dilution of CYP4Z1 rabbit polyclonal (Cat# DF3590 Affinity biotechnology) was used to incubate the cells overnight on a refrigerated shaker (4°C, 80 RPM) and incubated with 1:2000 dilution of secondary antibody goat anti-rabbit IgG conjugated to Alexa fluor 488 (Cat # A11008 Life technologies) for 2 h at room temperature. Fixed cells were mounted in vectashield with DAPI.

### 2.11. RNA isolation and real-time RT-PCR

Total RNA was isolated from MCF10 and MCF7 cell cultures by using an Eastep Super RNA isolation kit (Shanghai, Promega Biological Products, China) according to the manufacturer’s instructions. Total RNA concentrations were measured with a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE). The cDNA synthesis was performed in a thermocycler GeneAmp® PCR System 9700 PE (Applied Biosystems, Foster City, CA), using a HiFi Script 1st strand cDNA synthesis kit (CW Biotech, China) with 1 μg of total RNA in a 20 μl reaction. This cDNA was diluted to 300 μl and stored at −80°C until real-time PCR analysis. Real-time PCR reactions for cDNA were performed using 5 μl cDNA and Ultra SYBR mixture with low ROX (CW biotech, China) according to the manufacturer’s instructions in a LightCycler (Applied Biosystems, Quant studio 6 Flex real time PCR system, San Francisco, CA) as explained by (Livak & Schmittgen, 2001). For quantitative real-time PCR, the values of relative target gene expression were normalized for YWHAZ housekeeping gene expression. Real-time PCR was used to assess expression of the following genes: SPCA1, IGF1R and Nfix. Primer-pairs used were as follows: YWHAZ, forward-5ʹ-GATGAAGCCATTGCTGAACTTG 3ʹ, reverse-5ʹ-CTATTGTGGGACAGCATGG 3ʹ (product length 229 bp); IGF1R, forward-5ʹ-CTTCTGTTTCTCCTCCGGC 3ʹ, reverse-5ʹ-ATAGTCGTCCGGATGTCGAT 3ʹ (product length 241 bp). SPCA1, forward-5ʹ-GGACCATACACTTGCCCGAGA 3ʹ, reverse-5ʹ-GGAGCATACACTTGCCCGAGA 3ʹ (product length 131 bp), and Nfix, forward-5ʹ-GGACAGGACAGCTGGATGTG 3ʹ, reverse-5ʹ-GGACAGGACAGCTGGATGTG 3ʹ (product length 241 bp).

### 2.12. Homology modeling and docking

In order to construct 3D structures of SPCA1/2, the amino-acid sequences of human SPCA1 and 2 were retrieved from the UniProt database [http://www.uniprot.org/uniprot/](http://www.uniprot.org/uniprot/), with corresponding accession number of P98194 and Q75185. Since both SPCA and SERCA belong to P2A-type Ca²⁺ ATPase family, the 3D crystal structure of SERCA; PDB ID: 2AGV, 2.40 Å resolution (Obara et al., 2005), using as template, was downloaded from RCSB Protein Data Bank [https://www.rcsb.org/](https://www.rcsb.org/). Sequence alignment between the target sequence and the template sequence was implemented as default parameters by ClustalW 2.0.10 program accessible from [http://www.ebi.ac.uk/Tools/clustalw2/index.html](http://www.ebi.ac.uk/Tools/clustalw2/index.html) (Chenna et al., 2003). One hundred different 3D structures of SPCA1/2 were built by MODELLER 9v10 program (Fiser & Sali, 2003) and were evaluated by PROCHECK program (Laskowski, MacArthur, Moss, & Thornton, 1993). The best models were selected to perform subsequent molecular docking with its two speculated ligands, Thapsigargin (Tg) and BPA.

Computational docking operation is a useful vehicle for investigating molecular binding interactions (Alam et al., 2015; iram et al., 2015; Nasir et al., 2017; Shaker et al., 2014). To simulate the binding modes of Tg and BPA to SPCA1/2, molecular docking studies were carried out by inserting inhibitors into small pocket which were originally occupied by Tg in the SERCA crystal structure (PDB ID: 2AGV). Molecular docking was performed by AutoDock 4.2 program with the application of the highest score and Lamarckian genetic algorithm principle (Morris et al., 1998). Parameters were set as follows: a population of 300 randomly placed individuals and a maximum of 2.5 × 10⁶ energy evaluations, which were used for 100 search runs for each ligand. The grid maps generated using the AutoGrid module were 40 × 40 × 40 points separated by 0.375Å along the X-, Y- and Z-axis. The conformation of ligand that showed the lowest binding energy was selected for subsequent analysis.

### 3. Statistical analysis

All experiments were replicated at least three times and averages plotted. All data were presented as mean values with standard error (SE). Data were compared using a one way analysis of variance (ANOVA), and post hoc comparisons were made using Bonferroni’s corrections. The significance level was set at p < 0.05. All statistical analyses were carried out using GraphPad Prism version 5 for Windows, GraphPad software, La Jolla, CA, USA.
4. Results

4.1. BPA-induced single-strand breaks (SSBs) DNA damage in target cell lines

DNA damage analysis in BV2, MDA-MB-231, MCF-10A and MCF-7 cell lines, measured at 3, 6, 24 and 48 h exhibited that a low-dose BPA (2–4 µg/mL) induced longest comet tail lengths (SSBs) at 6 h post treatment in all cell lines especially MCF-10A (Figure 1(c)). However in BV2 this damage was repaired within 24 h as shown in figure. In MDA-MB-231 cells, the damage induced by 2 µg/mL BPA showed a sharp increase at 3 and 6 h while in the 24 and 48 h groups the damage was largely repaired however a gradual increase could still be observed. In both breast cancer cell lines MDA-MB-231 and MCF-7 the damage induced by BPA could not be fully repaired. The observation in MDA-MB-231 could be due to the inability of these cells to repair the damage induced by BPA, since these cells are late stage breast cancer cell line. For all four cell lines, BV2, MDA-MB-231, MCF-10A and MCF-7, the temporal effect experiments showed the 6 h time point and 2–4 µg/mL of BPA concentration produced the highest DNA damage (SSBs) as measured by alkaline comet assay. Comet tail length of the electrophoresed DNA was used as a measure of the extent of DNA damage (Figure 1(e)).

4.2. BPA-induced SPCA1/2 production inhibition and IGF1R elevation

For NF-κB and pIκB, we replicated some of the results reported by other labs (Zhu et al., 2015) for inflammation induced by various BPA concentrations (data not shown) that were in the physiological range of low dose and showed highest DNA damage in our experiments at 2–4 µg/mL. We then proceeded to measure the SPCA1/2 and IGF1R protein production. For SPCA1/2 and IGF1R measurements, we used whole cell lysates from BPA treated breast cancer cell line MCF-7 and subsequent control cell line MCF-10A to test our hypothesis of BPA-induced tumorigenesis. The results are shown in Figure 2(a–d). In MCF-10A cells (Figure 2(a)) SPCA1/2 protein production showed a reduction of 99.7% compared to mock. However in MCF-7 cells an increase of 482% was recorded at 4 µg/mL. When we measured IGF1R production in MCF-10A cells (Figure 2(c)) an increase of 68% and 79% at 2 and 4 µg/mL BPA concentrations was recorded, respectively. Conversely in breast cancer cell line MCF-7 a completely opposite trend was observed (Figure 2(d)) where a 67% and 80% decrease was recorded, respectively. The trends recorded for SPCA1/2 and IGF1R were almost completely opposite in normal and breast cancer cell lines.

4.3. BPA-induced mRNA expression modification of SPCA1/2 and IGF1R

We compared our western blot data through RTPCR analyses and the results are shown in Figure 3. The outliers were not included in this graph. The internal housekeeping control used was YWHAZ. Low-dose BPA (2–4 µg/mL) did not seem to affect the gene expression of SPCA1/2 and IGF1R in MCF-10A cells (Figure 3(a–c)) but a significant SPCA1/2 increase (Figure 3(b)) was recorded at 4 µg/mL in MCF 7 cells, while a 3.7% and 87% IGF1R increase (Figure 3(d)) was recorded in MCF7 cells, respectively.

4.4. BPA-induced CYP4Z1 detection in MCF-7 and MCF-10A cell lines

A 6 h BPA treatment induced quantifiable DNA damage. When MCF-10A and MCF-7 cells were treated with BPA, immediately fixed on glass cover slips and probed for CYP4Z1 detection, we noticed an increase (but not significant increase) in MCF-10A cells compared to mock. This was a clear indication that CYP4Z1 was being induced in these cells (Figure 4(a–c)).

4.5. Homology modeling of SPCA1/2 and docking with BPA

For SPCA1 and SPCA2, the majority of contacts identified were hydrophobic interactions (Figure 5). Compound Thapsigargin (Tg) was surrounded by a hydrophobic pocket which was composed of Leu264, Leu265, Gln268, Leu269, Tyr272, Leu707, Ile711, Leu771, Ile776, Thr772 and Leu780 of SPCA1, and Arg256, Val260, Ile769, Asp772, Pro829, Arg837 and Thr838 of SPCA2. Carbonyl groups of Tg made three hydrogen bonds with——NH2 of Arg837 and hydroxyl group of Thr838 for SPCA2 (Figure 5(z)). The predicted binding energy and inhibitor constant of Tg and BPA with SPCA1 and SPCA2 is shown in Table 1.
The central hydrophobic part of BPA was in close contact with Leu265, Leu269, Leu707, Ile711 and Leu771 of SPCA1, and Arg256, Val260, Ile306, Leu308, Leu765 and Ile769 of SPCA2. In addition, the hydroxyl groups of BPA accepted two hydrogen bonds from the backbone amide of Leu707 and the hydroxyl group of Ser769 for SPCA1, and one hydrogen bond from the backbone amide of Ile306 for SPCA2 (Figure 5(b)).

5. Discussion

BPA is a widely occurring environmental toxin and an endocrine disruptor that has been long known to cause DNA damage. The BPA 3, 4-quinone forms an adduct at the N7 position of guanine in the DNA (Edmonds et al., 2004; Jung & Suh, 2015). But DNA repair machinery manages to efficiently repair most of the SSBs; however some of the severe damages such as double-strand breaks (DSBs) can persist in the cell to induce mutations (Branzei & Foiani, 2008). Besides several group and cohort studies done in mice the carcinogenic potential of BPA has long been realized but only raising the risk level to a class 2B carcinogen (Health Council of the Netherlands, 2019; Yamasaki et al., 2003). For several years various substitutes of BPA have been introduced in the market to reduce the health risk but research soon caught up to show that both BPA and BPAF (C15...
H$_{10}$F$_2$O$_2$) were possibly involved in enhanced carcinogenicity and neurotoxicity (Brede et al., 2003). Most recently, a BPA mediated misregulation of the antisense transcript, long non-coding RNA called HOTAIR, was suggested to play a vital role in breast cancer incidence. The tests performed in-vitro and in-vivo highlighted the risk factor of BPA yet again (Bhan et al., 2014).
scientific investigations the actual molecular link between BPA and cancer remained an elusive phenomenon (Bhan et al., 2014; Jenkins et al., 2009; Lee et al., 2013). The Golgi localized secretory pathway calcium ATPase 1/2 (SPCA1/2) is already known to play a role in the anterograde transport of proteins to the surface of the cell (Lissandron et al., 2010). Any compound that inhibits SPCA1/2 would be expected to inhibit the SPCA1/2 mediated intracellular vesicular transport and disrupt the cytosolic calcium homeostasis (Mascia et al., 2012). The in-silico homology modeling data for SPCA1/2 suggested inhibition of target protein by BPA, verifying the observations that BPA-induced SPCA1/2 inhibition could dysregulate the anterograde trafficking of IGF1R transportation.

Cancer cells have been recognized to frequently exploit the insulin like growth factor (IGF) signaling pathway to mediate development, cell proliferation and survival (Malaguarnera & Belfiore, 2014). Thus the hypothesis of IGF1R dysregulation via BPA-induced SPCA1 interaction (Jalal et al., 2017) was tested in this research. Other signaling pathway activations such as MAPK and JNK have already been shown in post-BPA exposure environments (Kurebayashi, Betsui, & Ohno, 2003; Zhu et al., 2015). BPA-induced DNA damage (SSBs)

The DNA damaging effect of duration of exposure on target cell lines BV2, MDA MB231, MCF-7 and MCF-10A was measured. Results shown in Figure 1(a–e) indicated that BV2 and MCF-10A cells that had normal repair machinery, could begin to reduce the overall burden of BPA-induced DNA damage within 24–48 h (Figure 1(a,c)) while the cancer cell lines MDA MB231 and MCF-7, having faulty repair machinery, demonstrated a trend of accumulation of DNA damage even after 24–48 h (Figure 1(b,d)). The damage in BV2 cells showed that total damage burden went down at 24 and
48 h (as shown in Figure 1) but the high concentration exposure of cells to BPA (8 μg/mL) indicated a decreased damage possibly due to increased cytotoxicity and mortality (data not shown). The overall damage measured at 4μg/mL concentration showed a decrease in BV2 and MCF-10A cells but the cancer cell lines MDA MB231 and MCF-7 were not affected by this concentration at 24–48 h (Figure 1(c,d)). For MCF-10A and MCF-7 cell lines, the highest DNA damage could be recorded at 6 h post-treatment, which was hence used as the time point for all succeeding experiments. The most challenging concentration of BPA was also measured to be 2–4 μg/mL. Hence, a low-dose BPA concentration of 2 and 4 μg/mL and 6 h exposure time was selected for further experimentation.

5.1. Western blot analysis of the BPA-induced tumorigenic proteins
The western blot analyses of NF-κB, p-1kB (data not shown), SPCA1/2 and IGF1R were treated with mock, 2 and 4 μg/mL BPA concentrations. Our and other researchers’ results (Zhu et al., 2015)
indicate (data not shown) that inflammation pathway was activated at 2 µg/mL exposure of BPA, which has also been reported by other labs. As NF-κB protein production was elevated in control cell line MCF-10A at 2 and 4 µg/mL it was a confirmation that these low doses play a highly toxic role in the target cell lines. Concomitantly the p-IκB expression is reduced at 2 and 4 µg/mL in MCF-10A. Whereas the SPCA1/2 production is increased in MCF-7 cells but decreased in MCF-10A cells at 2 and 4 µg/mL, this shows a direct effect of BPA on protein production. However, our RTPCR data suggest that this inhibitory effect could be at post-translational level since we could not observe a significant change at the transcriptional level in MCF-10A cells (Figure 2(a)). Western blot data further indicated that IGF1R is elevated at 2 µg/mL in MCF-10A cells, compared to the background (Figure 2(c)); hence this dysregulation is a clear evidence of acquiring a tumorigenic phenotype, whereas the expression of IGF1R in MCF-7 cells is reduced (Figure 2(d)) when compared to mock.

5.2. RTPCR analysis of the mRNA expression of BPA-induced signaling proteins
To verify our findings of SPCA1/2 and IGF1R protein production we compared the results with real time qPCR data. Inflammation marker NFκB indicated that 2 µg/mL treatments in MCF-10A cells caused a 63% increase in protein transcription (data not shown). For SPCA1/2 we did not observe a significant change in protein expression with BPA treatment for MCF-10A cells (Figure 3(a)) but a significant increase was recorded in MCF-7 cells (Figure 3(b)). This indicated a protein production inhibition with BPA treatment but protein expression was unaffected. For IGF1R analysis we measured a 69% increase in protein production at 2 µg/mL in MCF-10A cells compared to 70% decrease in MCF-7 cells (Figure 2(c–d)) but only 40% increase with RTPCR analysis (Figure 3(c–d)). This was the strongest evidence in support of our hypothesis of BPA causing a significant change in the production and expression of breast cancer marker IGF1R in MCF-10A cells.

5.3. BPA-induced CYP4Z1 detection in MCF-7 and MCF-10A cell lines
Previously, CYP4Z1 has been shown (Rieger et al., 2004) to have significantly higher expression (4–5 folds) in breast carcinomas than in any other tissue. Our data indicates that low-dose BPA treated normal MCF-10A cells demonstrated a conditioned increase of CYP4Z1 production (Figure 4(b)). However, the BPA treated cancer cell line MCF-7 did not demonstrate any significant increase at 2 µg/mL but showed a drastic decrease at 4 µg/mL (Figure 4(c)). This early breast carcinoma marker is elevated by low-dose BPA exposure in MCF-10A cells.

5.4. Homology modeling
The in-silico homology modeling data for SPCA1/2 indicated that the central hydrophobic part of BPA was in close contact with Leu265, Leu269, Leu707, Ile711 and Leu771 of SPCA1, and Arg256, Val260, Ile306, Leu308, Leu765 and Ile769 of SPCA2 (Figure 5). Also, the hydroxyl groups of BPA accepted two hydrogen bonds from the backbone amide of Leu707 and the hydroxyl group of Ser769 for SPCA1, and one hydrogen bond from the backbone amide of Ile306 for SPCA2, suggesting inhibition of target protein due to BPA.

6. Conclusion
According to our results and existing literature low-dose BPA exposure can initiate tumorigenic events in three ways. (1) Direct interaction with DNA via adduct formation, thus binding covalently to the

|  | Binding energy (kcal/mol) | Ki nM | Binding energy (kcal/mol) | Ki µM |
|---|--------------------------|-------|--------------------------|-------|
| SPCA1 | -8.37 | 733.15 | -4.36 | 639.36 |
| SPCA2 | -8.91 | 293.2 | -4.53 | 475.19 |
DNA and increasing hypermutability and genomic instability, also aggravated by Ca\(^{2+}\)-induced oxidative stress and NF-κB mediated inflammation. (2) Direct functional inhibition of SPCA1/2 as demonstrated by in-silico analysis and additionally a production inhibition as demonstrated by Western analysis data which exhibit decreased SPCA1/2 production in MCF-10A cells when exposed to low-dose BPA 2–4 μg/mL but RTPCR data suggests that there was no change in mRNA expression of the SPCA1/2 gene. This production inhibition of SPCA1/2 leads to dysregulation of IGF1R, which needs Ca\(^{2+}\) for post-translational modification hence the modified receptor gets accumulated on the surface membrane and is possibly not cycled through the retrograde transportation mechanism. This process sets up sustained proliferation signaling while the downstream signaling via Ras/Raf/MEK/ERK pathway can further set up cell proliferation signaling cues. (3) Increased production of CYP4Z1 in the breast epithelial cell line MCF-10A. We further hypothesize that once IGF1R expression and production is increased due to BPA exposure, it could constitutively activate MAPK or JNK pathway (in the absence of a ligand) to upregulate cell proliferation (Lan, Lin, Yang, & Lin, 2015) but needs to be tested. Another interesting outcome of SPCA1/2 production inhibition could be the inability to maintain Ca\(^{2+}\) homeostasis within the Golgi Ca\(^{2+}\) stores.

**Article Highlights**

Low-dose nano-molar Bisphenol (A) exposure increases the risk of tumorigenesis in normal breast epithelial cell line MCF-10A in a three-step process: (i) induced DNA damage, (ii) cytochrome P450Z1 (CYP P4Z1) overexpression and (iii) SPCA1/2 inhibition that leads to IGF1R dysregulation.

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**Author contribution**

All authors contributed towards intellectual input, data collection, data generation, analysis and drafting of this article.

**Disclosure**

The authors report no conflict of interest in this work.

**Cover Image**

Source: Legend: Bisphenol A (BPA) at low, physiologically relevant dose of 8.73 and 17.47 nm can cause quantifiable DNA damage. BPA-induced SPCA1/2 inhibition, within Golgi apparatus, and thus disrupts the anterograde trafficking of IGF1R transportation. The central hydrophobic part of BPA interacts with Leu265, Leu269, Leu707, Ile711 and Leu771 of SPCA1, and Arg256, Val260, Ile306, Leu308, Leu765 and Ile769 of SPCA2. The in-vitro findings are supported by in-silico data to show that BPA-induced SPCA1/2 inhibition leads to IGF1R dysregulation and cytochrome P450Z1 (CYP4Z1) upregulation thus triggering the tumorigenic phenotypic change in normal breast epithelial MCF-10A cell line.

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