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Lestaurtinib induces DNA damage that is related to estrogen receptor activation

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ABSTRACT

A number of chemicals in the environment pose a threat to human health. Recent studies indicate estradiol induces DNA damage through the activation of the estrogen receptor alpha (ERα). Given that many environmental chemical compounds act like hormones once they enter the human body, it is possible that they induce DNA damage in the same way as estradiol, which is of great concern to females with the BRCA1 mutation. In this study, we developed an antibody-based high content method measuring γH2AX, a biomarker for DNA damage, to test a subset of 907 chemical compounds in MCF7 cells. The assay was optimized for a 1536 well plate format and had a satisfactory assay performance with Z-factor of 0.67. From the screening, we identified 128 compounds that induce γH2AX expression in the cells. These compounds were further examined for their γH2AX induction in the presence of an ER inhibitor, tamoxifen. After tamoxifen treatment, four compounds induced less γH2AX expression compared to those without tamoxifen treatment, suggesting these compounds induced γH2AX that is related to ERα activation. These four compounds were chosen for further studies to assess their ERα activating capability and *c-MYC* induction. Only lestaurtinib, a selective tyrosine kinase inhibitor, induced ERα activation, which was confirmed by both ERα beta-lactamase reporter gene assay and molecular docking analysis. Lestaurtinib also increased *c-MYC* expression, a target gene of ERα signaling, measured by the quantitative PCR method. This data suggests that lestaurtinib acts as a DNA damage inducer that is related to ERα activation.

Introduction

Endocrine disruptors, also known as environmental hormones, are environmental compounds that enter the body and disrupt the normal function of the endocrine system (Yilmaz et al., 2020). They have the potential to cause physiological defects and pathologies in developmental, reproductive, and immunological systems. Developing safety tests to assess the risks of environmental compounds requires an understanding of how compounds work in the body. For example, bisphenol A (BPA), which is one of the most common endocrine disruptors, binds to both estrogen receptors (ERs) (i.e., ERα and ERβ) and acts as an agonist for ERα and an antagonist for ERβ, respectively (Marino et al., 2012). In addition, some endocrine disruptors have carcinogenicity (Soto et al., 2010), where activated ERs induce oncogenes, including the *c-MYC* gene, and strongly stimulate mammary epithelial cells. Some environmental chemicals, such as benzo [a] pyrene and *N*-nitrosodimethylamine, which are found in tobacco smoke, cause DNA damage in cells (Ooka et al., 2016). DNA damage is highly associated with mutations in the genome, leading to the dysfunction of enzymes. As a result, cells are unable to undergo programmed cell death and then develop cancer.

Breast cancer is one of the most frequently diagnosed cancers in women. The cells of breast cancer patients often overexpress Human Epidermal Growth Factor Receptor 2 (HER2), a receptor known to promote cell proliferation (Ménard et al., 2000). HER2 expression is regulated by ERs. Once estrogen binds to ERs, it activates transcription to promote cell proliferation. Besides growth-stimulating activity, estrogen also has genotoxic potential at the physiological concertation (Morimoto et al., 2019; Pommier et al., 2022; Stork et al., 2016). BRCA1, which is strongly related to breast cancer, is responsible for the repair of

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this class of DNA damage (Sasanuma et al., 2018). This DNA damage induction is initialized by the activation of ERα, which triggers early transcription response (Carroll 2016). The topoisomerase 2 has been shown to regulate early transcription response by forming topoisomerase 2-DNA covalent complex (TOP2cc) at promoter regions as reviewed by Pommier et al (Pommier et al., 2022). The TOP2cc is a toxic reaction intermediate, and it is recognized as DNA damage by DNA repair systems in cells (Canela et al., 2019). Considering some environmental compounds are known to act like hormones, it is possible that they induce DNA damage in the same mechanism as estrogen. To evaluate the risk of environmental chemicals to ER-related organs (e.g., breast and ovary), there is an urgent need to develop an assay system that can assess ER-dependent DNA damage.

Several cell-based assays have been developed to identify the molecular mechanisms of the compounds. Some of these assays can address molecular mechanisms of compound action but are designed for low throughput formats. On the other hand, some assays are designed for a higher throughput format but can only measure a limited number of targets/pathways, such as the assay that detects chemicals that activate p53-dependent stress response (Ooka et al., 2022). To provide a more informative detection assay in multi-well plate format, we have developed high-content screening assays (Li et al., 2018; Li et al., 2019; Shahane et al., 2014). The high-content screening platform often employs cells expressing fluorescent-labeled proteins so that the kinetics and localization of the target protein can be readily monitored. Nevertheless, this class of assays requires engineered cell lines designed for specific assays.

Herein, we developed and optimized the antibody-based γH2AX assay to identify environmental chemicals that cause DNA damage using a high-content screening system in a 1536 well plate format (Nishihara et al., 2016b; Zhang et al., 2021). We used this assay to screen a subset of 907 compounds, including 127 previously-identified ERα activator, that showed biological activities in the previous Tox21 screening related to cancer signaling or DNA damaging (Huang et al., 2016). To determine whether the DNA damage induction depends on ERα, we examined the compounds in the absence or presence of tamoxifen, an ER inhibitor. The binding of the compounds to ERα was also examined by a reporter gene assay and molecular docking analysis. To further evaluate the carcinogenic effect of these compounds, we measured *c-MYC* expression after compound treatment using the quantitative-PCR (qPCR) method.

Materials and methods

Reagents

The test compounds dissolved in DMSO were cherry-picked from the Tox21 10K compound library. Bleomycin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Nine hundred and seven compounds were selected from the Tox21 10K compound library for this study. These compounds showed biological activities in at least one of the in vitro assays, including estrogen receptor, activator protein-1 (AP-1), antioxidant response element (ARE), caspase-3/7, H2AX, hypoxia-response element (HRE), Nuclear factor-κB (NF-kB), or p53 assay (https://tripod.nih.gov/tox21/pubdata) (Huang et al., 2016).

Cell culture

MCF7 cells (ATCC, Manassas, VA, USA) were cultured in Eagle's Minimum Essential Medium (ATCC) supplemented with 10 % FBS (Thermo Fisher Scientific, Waltham, MA, USA), 50 U/ml penicillin, 50 μg/ml streptomycin (Invitrogen, Waltham, MA, USA) and 0.01 mg/ml human recombinant insulin (Sigma-Aldrich).

ER alpha-UAS-bla GripTite™ cells (Invitrogen) were cultured in DMEM + Glutamax (Invitrogen) supplemented with 10 % Dialyzed FBS (Invitrogen), 0.1 mM NEAA (Invitrogen), 50 U/ml penicillin, 50 μg/ml streptomycin (Invitrogen), 80 μg/ml Hygromycin B (Invitrogen) and 100 μg/ml Zeocin (Invitrogen).

γH2AX immunostaining assay in a 1536 well plate format

MCF7 cells, suspended in assay medium containing phenol red-free DMEM supplemented with 2 % charcoal-stripped FBS (Thermo Fisher Scientific) and 50 U/ml penicillin, 50 μg/ml streptomycin (Invitrogen), were plated at 1,500 cells per well in 5 μL assay medium into 1536 well clear bottom/black wall plate coated with poly-ethylenimine (Aurora Biosciences, San Diego, CA, USA). The cells were treated with 23 nl of test compounds, bleomycin, a positive control, or DMSO, a vehicle control. After a 4-hour compound treatment, cells were fixed with 4 % paraformaldehyde for 20 min and then were incubated with a permeabilization/blocking/nuclear-staining solution (5 % BSA, 0.5 % Tween-20, 1 μg/ml Hoechst). Next, cells were treated with the anti-γH2AX antibody for 45 min, followed by incubation with an anti-mouse Alexa488 antibody for 30 min. The fluorescence intensities (490 nm excitation and 550 nm emission) of the assay plates were measured on the Operetta CLS High-Content Analysis System (Perkin Elmer, Waltham, MA, USA) with a 20× confocal objective. Images from each assay well were acquired and analyzed by Harmony High-Content Imaging and Analysis Software (Perkin Elmer). The number of nuclei counted in each well was measured to reflect the number of cells and the number of spot count measurements used to quantitate the γH2AX expression in each well. The data are presented as the number of foci per cell.

ERα beta-lactamase reporter gene assay

The ERα beta-lactamase reporter assay was carried out as described previously (Huang et al., 2015). Briefly, ER alpha-UAS-bla GripTite™ cells were resuspended in assay medium (phenol red-free DMEM supplemented with 2 % charcoal-stripped FBS, 0.1 mM NEAA, 1 mM sodium pyruvate, 50 U/ml penicillin, 50 μg/ml streptomycin) and dispensed at 20,000 cells/40 μL/well in 384 well black wall/clear bottom plates (Greiner Bio-One North America, Monroe, NC, USA). After assay plates were incubated at 37 °C for 5 h, the compound dissolved in DMSO or DMSO only was added to the assay wells. The assay plates were then incubated for an additional 16 h. The next day, 8 μL of LiveBLAzer™ (Thermo Fisher Scientific) detection mixture was added to each well, and the plates were incubated at room temperature in the dark for 1.5 h. Fluorescence intensity (460 and 530 nm emission and 405 nm excitation) was measured by the PHERAstar plate reader (BMG Labtech, Germany). The data were presented as the ratio of the 460/530 emission.

c-MYC expression measured by quantitative polymerase chain reaction (qPCR)

MCF7 cells were plated at 12,000 cells per well in 12-well plates. After 24 h of serum starvation in the assay medium, cells were incubated with the compounds for 6 h. After washing with PBS twice, cells from 3 wells were combined and their RNA were extracted using the RNeasy Mini Kit according to the manufacturer's instructions. RNA concentrations were determined with NanoDrop™ (Thermo Fisher Scientific). A total of 2 μg of total RNA from each sample was used to perform the qPCR with the RNA-to-cDNA Kit (Thermo Fisher Scientific). The total volume for each reaction for qPCR was 20 μL. The TaqMan™ gene expression assay (Thermo Fisher Scientific) was used to determine the expression level of *c-MYC* (TaqMan Assay ID: Hs00153408_m1). GAPDH was used as an internal control. The qPCR reactions (1 cycle at 95 °C for 10 min for Taq polymerase activation; 40 cycles at 95 °C for 15 s and at 60 °C for 1 min for annealing/extension) were run on a ViiA 7 Real-Time PCR System (Thermo Fisher Scientific) according to the manufacturer's instructions. Induction values were calculated using the following equation: Fold $= 2^{-\Delta\Delta Ct}$, where ΔCt represents the differences in cycle threshold numbers between *c-MYC* and GAPDH, and ΔΔCt represents

the relative change in these differences between with compound treatment and without compound treatment.

Data analysis

Analysis of compound concentration–response data was performed as previously described (Huang 2016). Briefly, raw plate reads for each titration point were first normalized relative to the positive control compound (bleomycin, 100 %) and DMSO only wells (0 %) as follows:

$$\%\,Activity = \left[\left(V_{compound} - V_{DMSO}\right)/\left(V_{pos} - V_{DMSO}\right)\right] \times 100$$

Where $V_{compound}$ denotes the compound well values, V_{pos} denotes the median value of the positive control wells, and V_{DMSO} denotes the median values of the DMSO-only wells. The half-maximal effective values (EC_{50}) for each compound and maximum response (efficacy) values were obtained by fitting the concentration–response curves of each compound to a four-parameter Hill equation. Compounds were designated as classes 1–4 according to the type of concentration–response curve observed. In the present study, DNA damage inducers were defined as compounds that induce γH2AX foci. Compounds with class 1.1, 1.2, 2.1, 2.2, and 3 curves were considered active, compounds with class 4 curves were considered inactive, and compounds with all other curve classes were defined as inconclusive. Data were analyzed and depicted using OriginPro 2015 (OriginLab Corp., Northampton, MA, USA) and GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA).

Molecular docking

The X-ray crystal structures of ERα (PDB code 1GWR) were retrieved from the Protein Data Bank. The original ligand (i.e., estradiol) was separated from the receptor structures before the molecular docking simulations. The 3D structures of compounds as ligands were downloaded from the PubChem website. The ERα receptor and ligands were prepared for docking simulation using AutoDockTools 1.5.7. The receptor and ligands were protonated. The receptor as a macromolecule has added the Kollman charges, while the ligands have added the Gasteiger charges. The molecular docking simulation was done using AutoDock Vina 1.2.0 (The Scripps Research Institute, La Jolla, CA, USA) (Trott et al., 2010). The best ligand conformation binding to the ERα LBD pocket was used for the further step of the analysis. The receptor-ligand complexes from the docking simulation were visualized using PyMOL 2.5.

Results

Development and optimization of γH2AX high content assay in a 1536-well plate format

To identify compounds that induce DNA damage, we developed, optimized, and validated a γH2AX immunostaining assay in a 1536 well plate format. A breast cancer cell line, MCF7, was selected for our screening model because of the high expression level of ERα, which is responsible for γH2AX induction by estradiol (Sasanuma et al., 2018). The critical issue for applying immunostaining to high content assay is the requirement of multiple washing steps, often causing detachment of cells from assay plates, and well-to-well variation in the assay readout. To ensure the cells attached well to the assay plates, we tested seven plate coating materials: Poly-D-Lysine (PDL), Polyethyleneimine (PEI), Type 1 Collagen, Human Fibronectin, Mouse Laminin, Basement Membrane Extract (BME), and PDL-BME. Among these coatings, PEI coating gave the highest number of remaining cells with fewer cell clumps after the immunostaining process (Supplemental Fig. 1). Therefore, PEI coating was selected for the γH2AX immunostaining assay. To reduce the steps in the assay process, we combined the permeabilization, blocking, and DNA staining procedures into one step to increase the probability of cell attachment to the assay plates (Supplemental Fig. 2). To validate the optimized assay performance, we plated MCF7 cells into a 1536-well plate and treated them with bleomycin, a known DNA damage inducer. The nuclei and the γH2AX were displayed in blue and green, respectively, for γH2AX foci counting (Fig. 1A). The total number of γH2AX foci in the nucleus area was measured. Using these parameters, we calculated the number of γH2AX foci per cell in each well to measure the DNA damage. In Fig. 1B, bleomycin increased the number of γH2AX foci in a concentration-dependent manner in a 1536-well plate, indicating DNA damage was induced after bleomycin treatment. The assay performance parameters, including signal-to-background ratio (S/B ratio) > 2, coefficients of variation (CV) < 10 %, and Z-factor > 0.5, are regarded as being of sufficient quality for primary screening. The γH2AX immunostaining assay performed well in 1536-well formats using bleomycin, with S/B ratio of 6.38, coefficient of variation (CV) of 10.1 %, and Z′ factor of 0.62.

Identification of γH2AX inducer using high content imaging analysis

Based on the results of previous Tox21 screenings, 907 compounds were selected from the Tox21 10K compound library for this study. The imaging-based γH2AX assay was used to screen these 907 compounds for their γH2AX induction. The assay performed well in the screening, with the average CV of 14.85 ± 5.77 %, S/B ratio of 5.53 ± 2.02, and Z′ factor of 0.32 ± 0.14, respectively. From the screening, 128 compounds with EC_{50} ranging from 0.11 nM to 68 μM were identified as potential DNA damage inducers based on efficacy > 20 %. As expected, several known DNA damage inducers, such as topotecan, actinomycin D, etoposide, and teniposide, were identified as γH2AX inducers (Fig. 2). These 128 compounds were re-cherrypicked for further study.

Identification of compounds that induce phosphorylation on H2AX depending on ERα

To test whether the compounds induced γH2AX that is related to ERα activation, we treated the cells with these 128 compounds in the presence or absence of tamoxifen, an ER inhibitor. Four out of the 128 tested compounds reduced γH2AX induction in the presence of tamoxifen compared to the cells without tamoxifen treatment based on a potency cut-off (>3 fold), implying the compounds might have induced DNA damage that is related to ERα activation. Among these four compounds, three are known topoisomerase inhibitors: topotecan, mitoxantrone, and rubitecan (Fig. 3A-C). These three compounds induced less γH2AX expression in the presence of tamoxifen. Lestaurtinib, known as CEP701, was identified as a potential novel γH2AX inducer that is related to activation of ERα (Fig. 3D) because tamoxifen significantly inhibited lestaurtinib-induced γH2AX, suggesting its γH2AX inducing activity was dependent on ERα activation.

Effect of γH2AX inducers on ERα activity

To investigate the relationship between these γH2AX inducers and ERα activity, we employed the ERα beta-lactamase reporter gene assay to assess the effect of these four compounds on ERα activation (Huang et al., 2015). As shown in Fig. 4A, lestaurtinib significantly increased ERα activation and mitoxantrone had a very weak effect on ERα activation, whereas topotecan and rubitecan had no effect on ERα activation. To further evaluate the potential binding site and the orientation by which lestaurtinib can directly bind to ERα, molecular docking analysis was used to predict the binding mode of all four compounds. As these four compounds were expected to be ERα agonists, the agonist-containing ERα form (PDB ID: 1GWR) was chosen to estimate the binding site of chemicals in the ERα LBD pocket. Lestaurtinib can be precisely docked into the ERα LBD pocket and shares the same molecular interaction sites, such as Glu 353, Agr394, and His524, as the positive

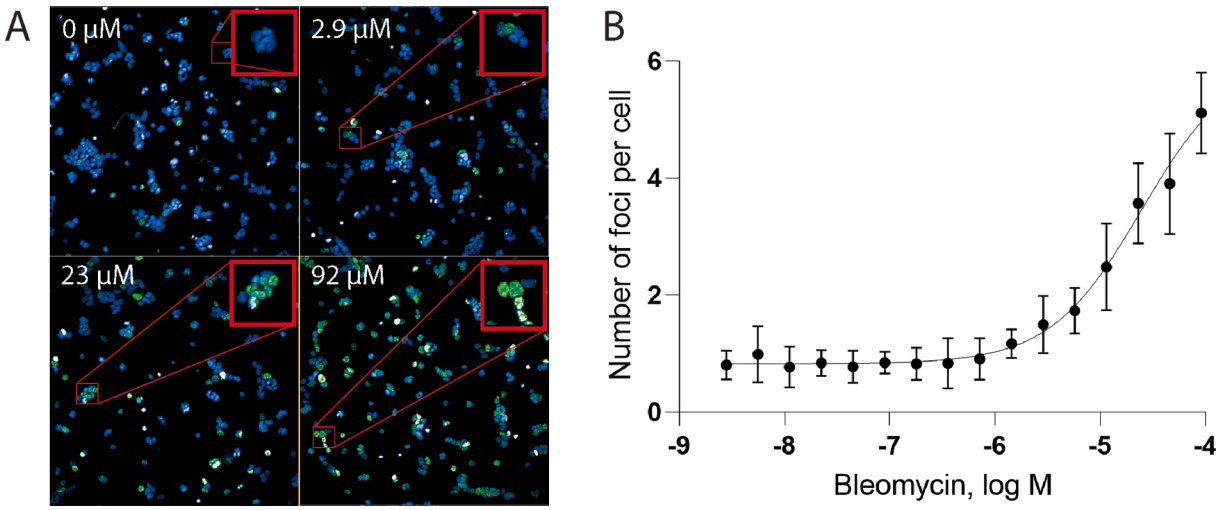

Fig. 1. γH2AX induction by bleomycin in MCF7 cells in a 1536 well plate. Cells were treated with bleomycin at 0, 2.9, 23, or 92 μM for 4 h. (A) Representative images show nuclei (blue) with γH2AX puncta (green). The red squares show zoomed images of representative nuclei. (B) Bleomycin concentration-dependently increased γH2AX induction. Error bars represent SD of three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

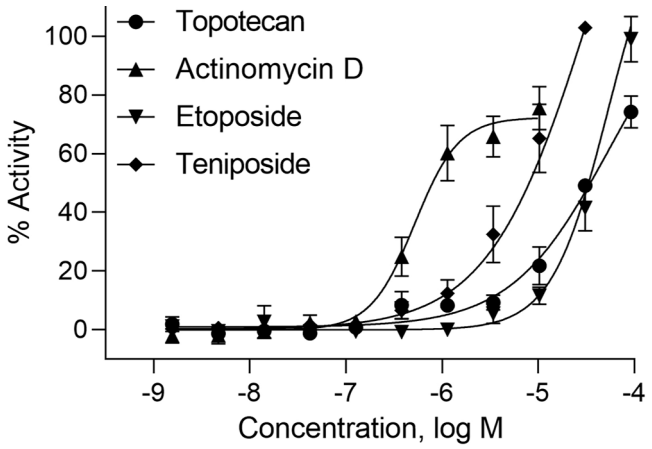

Fig. 2. Concentration-response curves with known DNA damage inducers for γH2AX induction. Cells were treated with the indicated compounds for 4 h. The Y-axis represents the γH2AX induction after compound treatments. The activity (%) of each compound was calculated based on the positive control compound, bleomycin at 92 μM, as 100 %. Error bars represent SD of three independent experiments.

control estradiol (Fig. 4B and Fig. S3A). However, the anticipated binding affinity of lestaurtinib to this active pocket is 10-fold lower than that of estradiol, which may help to explain the lower activity of lestaurtinib to activate ERα in the reporter gene assay compared with estradiol activity. Although a reporter gene assay detected minimal induction of ERα, mitoxantrone was also bound to the ERα active LBD pocket (Fig. S3B). In addition, the optimum posture of the other two topoisomerase inhibitors, topotecan, and rubitecan, was revealed to be outside of the active LBD pocket in the docking (Fig. S3C-D), despite the docking mode demonstrating that they interact with Agr394 of ERα from the outside of the pocket (Fig. S3C-D). These findings suggest that lestaurtinib induced DNA damage via ERα activation, but the other three compounds cause DNA damage independently of ERα activation.

Effect of lestaurtinib on c-MYC expression

To further explore the relationship between DNA damage and carcinogenicity of compounds, we measured *c-MYC* expression after treatment of γH2AX inducers since *c-MYC*, one of ERα's downstream genes, has been strongly linked to carcinogenesis. It is also known that estradiol induces the expression of *c-MYC*, which may be linked to potential carcinogenicity (Wang et al., 2011). Both estradiol and lestaurtinib stimulated *c-MYC* expression, and the increased expression was suppressed by tamoxifen (Fig. 5). However, mitoxantrone did not induce *c-MYC* expression. These data suggest that lestaurtinib can activate ERα, leading to the over-expression of *c-MYC* in estrogen-responding cells, which raises the probability of cancer occurring in ER-related organs, such as the breast and ovary.

Discussion

In this study, we applied the high-throughput and high-content imaging method to detect the compounds that induced γH2AX expression that is related to ERα activation. This imaging-based γH2AX high content assay in MCF7 cells has been successfully optimized into a 1536 well plate format. From the screening, 128 compounds were identified as γH2AX inducers, and these compounds were further tested in the same assay in the absence or presence of tamoxifen, an ER inhibitor. Four compounds showed a decrease in γH2AX expression after tamoxifen treatment, but only lestaurtinib bound to ERα and activated ERα activity, as well as induced *c-MYC* expression, suggesting lestaurtinib may induce DNA damage and carcinogenesis via ERα activation.

We developed a high-content screening method using sequential antibody treatment. This method gives more flexibility in both assay design and target choice since this method does not require reporter gene-integrated cells or commercially developed assay kits. One of the difficulties of applying immunostaining to HTS is that immunostaining requires many procedures, including washing steps. To reduce the number of experimental steps, we aimed to combine blocking, permeabilization, and DNA staining steps into one step. Triton X-100 is often used to permeabilize cells so that the antibodies have access to the nuclear protein. The blocking step usually requires 30 min at room temperature or overnight at 4 °C. Triton X-100 is a strong detergent, and immunostaining could not be done properly after 30 min of incubation with Triton X-100 in our assay condition. We applied a relatively gentle detergent, Tween-20, instead of Triton X-100 (Johnson 2013). We found that cells could be stained with anti-γH2AX antibody properly after 30 min of tween-20 treatment, which enabled us to combine permeabilization with the blocking process. Since DNA is already accessible to the antibody, it can be easily stained by Hoechst. Combining these

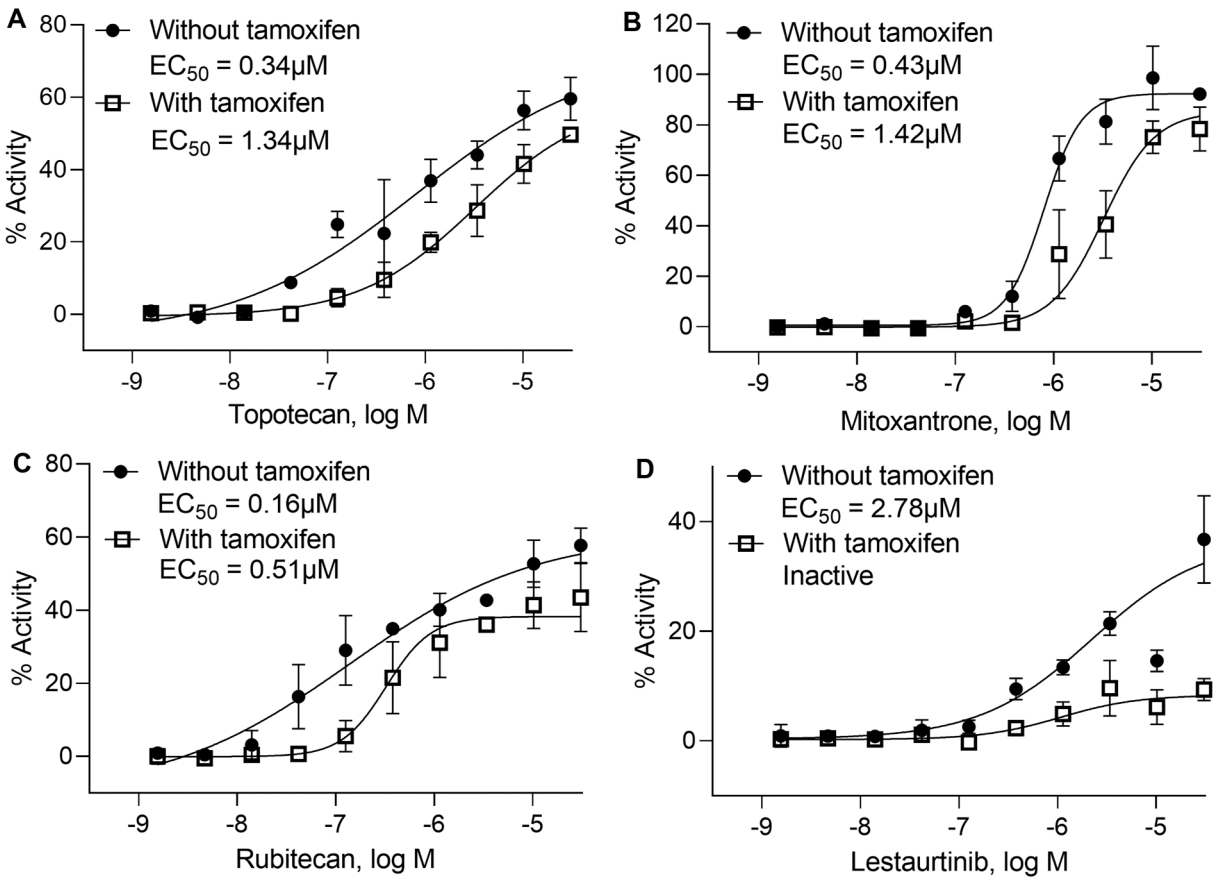

Fig. 3. Concentration-response curves of γH2AX inducers in the presence or absence of tamoxifen. Cells were treated with topotecan (A), mitoxantrone (B), Rubitecan (C), and lestaurtinib (D) for 4 h in the absence or presence of tamoxifen. The Y-axis represents the γH2AX induction after compound treatments. The activity (%) of each compound was calculated based on the positive control compound, bleomycin, as 100%. Error bars represent SD of three independent experiments.

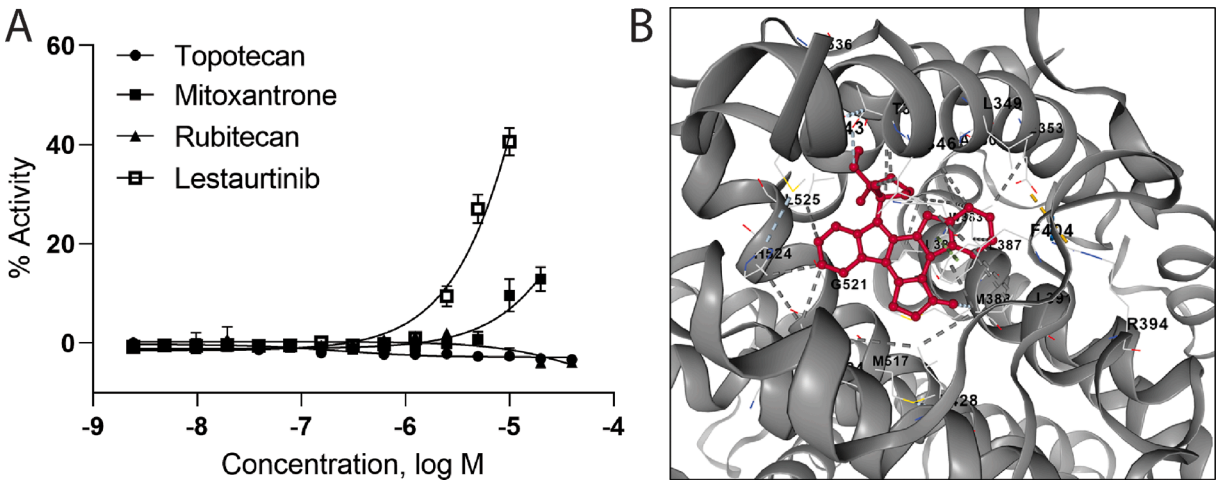

Fig. 4. Effect of compounds on ERα activity. (A) The cells were treated with the indicated compounds and tested for their ERα activation for 18 h. The Y-axis represents the ERα activation after compound treatments. The activity (%) of each compound was calculated based on the positive control compound, estradiol, as 100%. Error bars represent SD of three independent experiments. (B) Molecular docking result of lestaurtinib in the ligand binding domain of ERα.

three steps significantly reduces the experimental steps. Moreover, it also eliminates washing steps after each process (Supplemental Fig. 2). Methanol is another option for cell fixation, and it can also disrupt the nuclear membrane. Therefore, after fixation with methanol, nuclear protein can be stained with antibodies without permeabilization. However, since methanol is highly volatile, the assay plates need to be processed quickly, which is not suitable for the assay in the 1536 well plate format. Another issue of applying immunostaining to HTS is that the adhesion of cells is not robust during the experiment. To achieve better cell adhesion, we tested several plate coatings and found PEI coating dramatically improved cell adhesion after the experimental procedures (Supplemental Fig. 1). Each cell type may have its own

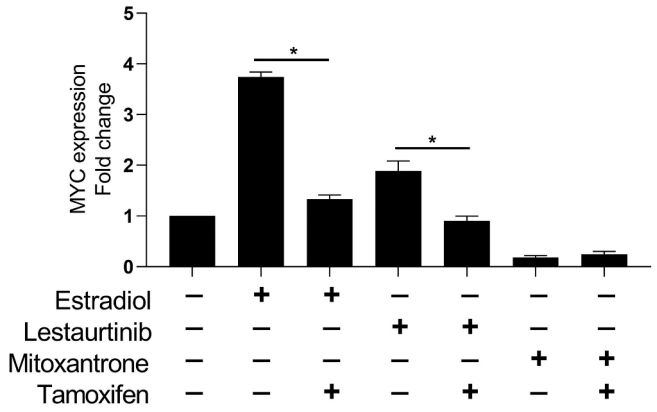

Fig. 5. Effect of compounds on *c-MYC* expression. Cells were treated with the 10 nM estradiol, 10 μM lestaurtinib, or 10 μM mitoxantrone in the presence or absence of 1 μM tamoxifen for 6 h after overnight serum starvation. Error bars represent SD of three independent experiments.

suitable coating method. Thus, coating optimization is required when testing different cell types in the high content screening.

Currently, several methods have been used to detect DNA damage. Single-cell gel electrophoresis (SCGE) (i.e., comet assay) is one of the most common assays used for DNA damage detection (Hirota et al., 2022). Cells were embedded in agarose gel on a glass slide after compound treatment. Following the lysis of the cells, an electric field is applied, leading to migration of DNA according to its size. Because migration is faster in smaller DNA, the distance of DNA migration increases when DNA is damaged. Therefore, measuring the distance of DNA migration provides the amount of DNA damage. Recently, SCGE has been optimized for 96 well and 384 well plate formats using the CometChip platform (Li et al., 2022; Sykora et al., 2018). Although SCGE is suitable for DNA repair kinetics studies due to its high sensitivity, it requires an engineered assay plate and detector for a high throughput format. The micronucleus assay is also used to detect DNA damage in high-content screening platforms (Nishihara et al., 2016a). Micronuclei are small nuclear bodies that are formed generally in the S or G2/M phase as a result of abnormal DNA synthesis, chromosome fragments, chromosome breakage, or chromosome loss due to mitotic disruption. Although this method has been optimized for a 384 well plate format and could also be optimized for the 1536 well plate format, ER-related DNA damage is observed in the G1 phase (Sasanuma et al., 2018). Therefore, we did not apply the micronucleus assay in this study. Another method used to detect DNA damage is the quantification of DNA damage response genes. A homogeneous assay system to detect the amount of γH2AX has also been developed using Homogeneous Time-Resolved Fluorescence technology (PubChem AID:1224845) (Kim et al., 2021). This assay is designed as a homogeneous assay and does not require washing steps, which is suitable for high throughput screening in a 1536 well plate format. However, MCF7 cells showed high background, and the induction of DNA damage could not be detected by this method in our assay condition (data not shown). The γH2AX immunostaining assay developed in this study performed well in 1536-well plates and can be used to screen a large library of environmental chemicals.

In the current study, four γH2AX inducers showed less γH2AX expression after tamoxifen treatment compared to without tamoxifen treatment, indicating they induce DNA damage that is related to ERα activation. However, among these four compounds, only lestaurtinib showed ERα activation. The other three compounds that are topoisomerase inhibitors did not activate ERα. Etoposide, a well-known topoisomerase inhibitor, was also tested in this assay. It showed slightly less amount of γH2AX in the presence of tamoxifen (efficacy: 61.8 % without tamoxifen, 39.8 % with tamoxifen). However, etoposide as well as other three topoisomerase inhibitors (Fig. 3A-C) did not activate ERα in the reporter gene assay (data not shown). The catalytic activity of topoisomerase is to induce DNA break to resolve DNA catenation caused by DNA synthesis or RNA transcription, followed by re-ligation of DNA (Bergant Loboda et al., 2020). Topoisomerase inhibitors stabilize topoisomerases on the DNA break site and prevent re-ligation process, leading to the increase of DNA damage. Therefore, the toxicity of topoisomerase inhibitors depends on their catalytic activity. Since tamoxifen has the potential to inhibit the catalytic activity of topoisomerase 1 and 2, the DNA damage caused by this class of compounds might be reduced by tamoxifen (Larosche et al., 2007).

Although molecular docking study reveals that both lestaurtinib and mitoxantrone can bind to ERα, only lestaurtinib significantly induced ERα in the reporter gene assay. Regarding the lower activation of ERα by mitoxantrone, we observed that mitoxantrone has a Met522 interaction site when docking to the agonist form of the ERα LBD pocket. Both estradiol and lestaurtinib do not have this interaction site, whereas 4-hydroxytamoxifeninteracting with the ERα antagonist form has this interaction site. Therefore, we hypothesize that the interaction of mitoxantrone with Met522 of the ERα LBD pocket leads to the instability of the active ERα conformation, which results in minimal detectable ERα activation.

Our study identified lestaurtinib as an ER-related DNA damage inducer. Lestaurtinib is a selective tyrosine kinase inhibitor for an acute myeloid leukemia patient with an FMS-like tyrosine kinase 3 (FLT3) mutant (Knapper et al., 2006; Levis et al., 2011). It inhibits the autophosphorylation of FLT3, leading to the induction of apoptosis in tumor cells overexpressing FLT3. Lestaurtinib also inhibits other kinases such as JAK2 (Hexner et al., 2008). Although lestaurtinib has been widely studied for its activity and is undergoing phase 3 clinical trials, its relationship with ERs has not been studied well. Lestaurtinib was reported to enhance the ability of the Poly (ADP-ribose) polymerase 1 inhibitor, AG14361, in breast cancer cells (Vazquez-Ortiz et al., 2014). Estradiol enhances the role of PARP inhibitors by stimulating the release of nitric oxide, which subsequently decreases the expression of BRCA1 in MCF7 (Zhou et al., 2021). It might be possible that lestaurtinib similarly decreases BRCA1 function and induces ERα-dependent transcription at the same time, leading to DNA damage in breast cancer cells as a result of a failure in removing TOP2cc. This study suggests that it may pose a risk to use lestaurtinib in females with BRCA1 mutation. However, because the working concentration of lestaurtinib is around 1 nM as a kinase inhibitor used in clinics and our study indicates lestaurtinib induces DNA damage at around 3 μM, it may not cause concerning amount of DNA damage in clinical usage. There might be a need to test the relationship among lestaurtinib, ERs and BRCA1 mutations in the future.

c-MYC is a proto-oncogene that responds to many activated growth factor receptors and the ERα signaling pathway (Chi 2012). Its expression is highly related to both breast cancer proliferation and breast cancer prognosis (Liao et al., 2000). A number of environmental compounds show a genotoxic effect, and they might promote cancer. In this study, Lestaurtinib was identified as a DNA-damaging *c-MYC* inducer through ER activation. This three-tier approach (i.e., DNA damage detection, ERα induction, and quantification of *c-MYC* expression) showed convincing results for evaluating compound genotoxicity caused by ER activation and provided an experimental approach to assess compound carcinogenicity. Our study also proposes the possibility of the use of *c-MYC* expression testing in qHTS methods to evaluate potential carcinogenic compounds in parallel with another genotoxicity testing.

Conclusion

In the current study, we used an imaging-based γH2AX assay to detect DNA damage that may be via ERα activation in MCF7 cells. This imaging-based γH2AX assay was developed and optimized into a 1536 well plate format. The assay worked well, with a Z-factor of 0.67, and we identified several known and novel γH2AX inducers in the screening.

The potential γH2AX inducers were further studied for their ability to activate ERα and induce *c-MYC* expression. From this study, lestaurtinib was identified as an ERα-related DNA damage inducer. Due to the high flexibility of the assay design, the immunostaining method optimized in this study will help identify environmental chemicals that induce biological events of interest in the future.

CRediT authorship contribution statement

Masato Ooka: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft and editing. **Shu Yang:** Methodology, Data curation, Visualization, Writing - review and editing. **Li Zhang:** Methodology. **Kota Kojima:** Methodology. **Ruili Huang:** Formal analysis, Writing - review & editing. **Kouji Hirota:** Conceptualization, Supervision, Investigation, Writing - review & editing. **Shunichi Takeda:** Conceptualization, Investigation, Supervision, Writing - review & editing. **Menghang Xia:** Conceptualization, Investigation, Methodology, Supervision, Writing - review & editing.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crtox.2022.100102.

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