The Transcriptome Induced by Bazhen Decoction and its Function in G-quadruplex Resolving and Telomere Maintenance in Progeroid Cells

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Abstract

Background and Purpose: The Bazhen decoction is one of the most extensively used Traditional Chinese medicine (TCM) prescriptions for anti-aging. However, due to the complicity of the components, the pharmacological mechanism of Bazhen decoction is still limited. Experimental approach: We applied RNA sequencing and transcriptome analysis to get the full view of the signaling pathways regulated by Bazhen decoction in the wild type cell background. By using the progeroid cells derived from Werner syndrome mice, we applied Western blot, Immunofluorescence, flow cytometry and telomere FISH to verify the transcriptome data. Key Results: The transcriptome profile revealed that Bazhen decoction might systematically regulate multiple anti-aging pathways, including stem cell regulation, protein homeostasis, cardiovascular function, neuronal function, anti-inflammation, anti-DNA damage induced stress, DNA helicase activity and telomere lengthening. We found that multiple DNA helicases and telomere regulating proteins were up-regulated by Bazhen decoction, which promoted the resolving of G-quadruplex (G4) structure, and facilitated DNA replication and telomere elongation. These improvements also endowed the cellular resistance to DNA damages induced by replication stress. Together these data suggest that Bazhen decoction facilitate G4 resolving and telomere maintenance, which might contribute to the longevity sustaining properties revealed by transcriptome profile. Conclusions & Implications: Our data revealed a new strategy for recovering the pharmacological signature pathways for TCM, which could help the clinical precision medicine of TCM. By applying transcriptome in TCM-treated normal cell, we tried out a systematic analysis for dissecting the molecular mechanism of complicated TCM prescription in the normal genetic background.

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Bulleted point summary:

- The transcriptome profile revealed that Bazhen decoction might systematically regulate multiple anti-aging pathways, including stem cell regulation, protein homeostasis, cardiovascular function, neuronal function, anti-inflammation, anti-DNA damage induced stress, DNA helicase activity and telomere lengthening.
- Bazhen decoction could up-regulate multiple DNA helicases and telomere regulating proteins, promote the resolving of G-quadruplex (G4) structure, and facilitate DNA replication and telomere elongation.
- Bazhen decoction could endow the cellular resistance to DNA damages induced by replication stress.
- Our data revealed a new strategy for recovering the pharmacological signature pathways for TCM, which could help the clinical precision medicine of TCM.

Abstract

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Key Words: Bazhen decoction, transcriptome, DNA helicase, telomere, G-quadruplex

Introduction

The traditional Chinese medicine strategy for body wellness and longevity focused on the balancing of Yin and Yang, or Xue (blood, nutrient) and Qi (energy). This anti-aging strategy applies to the prescription of Chinese medicine and generates varies classical prescriptions for treatment of aging-related disease and sustaining longevity. One of the most extensively used prescriptions for longevity is the Bazhen decoction. Bazhen decoction is composed of eight herbal medicine, including Panax ginseng, Radix rehmanniae praeparata, Radix paeoniae alba, Ligusticum wallichii, Poria cocos, Angelica sinensis, Rhizoma Atractylodis

Macrocephalae, and Licorice. Bazhen decoction is the combined prescription of Sijunzi and Siwu decoction, and is famous for enhancing both Qi and Xue, and balancing Yin and Yang. Thus, it is the one of the extensively used prescription for the intervention of sub-health status, and the prevention of aging-related degenerative diseases [1-4].

It has been revealed that Bazhen decoction was effective in the treatment of 5-fluorouracil-induced anemia in mice. It could promote the proliferation and differentiation of bone marrow cells, elevate the transcription of EPO mRNA, increase the red blood cell count and the hemoglobin concentration [5]. The similar effect of Bazhen has been found in the bone marrow depression induced by cyclophosphamide in mice, by promoting the proliferation of hematopoietic progenitor cell and secretion of hematopoietic growth factor [6]. It has also been shown that Bazhen decoction combined with sequential treatment of chemotherapy on acute lymphoblastic leukemia patients with deficiency of Qi and Yin could protect the hematopoietic system from damages induced by chemotherapy, and decrease the incidence of nausea and vomiting, liver and kidney injury [7]. Together these data suggest the function of Bazhen decoction in promoting hematopoietic regeneration.

It has also been found that Bazhen decoction administration could decrease acetaminophen-induced liver injury marker, maintain the activity of anti-oxidative factors, and depress the expression of pro-inflammatory factors. These data suggest that Bazhen decoction protect against acetaminophen induced acute liver injury by inhibiting oxidative stress, inflammation and apoptosis in mice [8]. The modification of Bazhen decoction (Huangqi Bazhen decoction) has been used to interfere the chemotherapeutic intestinal mucositis induced by capecitabine in mice. The results showed that the intestinal injury (villus height shortening, crypt destruction, and apoptosis) was reversed by Bazhen decoction, and the weight loss and diarrhea during capecitabine treatment were reduced [9]. These data suggest that Bazhen decoction could prevent tissue damages induced by toxic chemicals, such as chemotherapy drugs. The Bazhen decoction has been also apply to the treatment of premature ovarian failure induced by D-galactose in rats, it could up-regulate the E2 and X-linked inhibitors of apoptosis protein (XIAP), and prevented the apoptosis of oocyte and granulosa cells [10].

Together these data reveal the promising application of Bazhen decoction in prevent tissue damage and aging-related tissue degeneration. However, due to the complicity of the decoction components, the full image of the pharmacological basis of Bazhen decoction is still unclear, which hinder the precise clinical application, especially for complicated aging related degenerative diseases. The further dissection of the molecular pathways regulated by Bazhen decoction might provide the molecular pathways for the evaluation of treatment effect, and provide readouts for more precise symptoms and timing for the clinical application of Bazhen decoction on aging related diseases.

The hallmarks of aging include the attenuation of stem cell capacity, abnormal intercellular communication, abnormal mitochondrial function, increased chromosomal instability, altered epigenetic modifications, decreased protein homeostasis, decreased telomerase activity, and abnormal telomere function [11].

Telomere is at the end of eukaryotic chromosomes and composed of repeat DNA sequence TTAGGG and shelterin protein complex. Telomere is essential in maintaining the integrity of chromosomal DNA, and preventing chromosome ends from being recognized as DNA damage [12, 13]. Telomere length is mainly maintained by telomerase, or by a class of mechanisms referred to as alternative lengthening of telomere (ALT) [14]. In the absence of telomere lengthening mechanism, telomere DNA will gradually shorten along with the cell division, the shortened telomere activates DNA damage responses, such as p53-regulated signaling and the inflammation response pathways, eventually induced cellular senescence, apoptosis, and organism progeroid diseases [15].

In addition to telomerase, DNA helicases are also required for proper replication and elongation of telomeric DNA. Telomeric DNA is rich in G and tends to forms G-quadruplex DNA (G4 structure), which requires helicases to unwind properly for further DNA replication [16]. DNA helicases are a large family of proteins that hydrolyze ATP to produce energy to unwind DNA double helix. The RecQ family of DNA helicases, such as Wrn, Blm, Recql4, are involved in DNA replication, recombination, DNA damage repair and telomere

maintenance, and play an essential role in maintaining chromosome stability [17]. Thus, the function of DNA helicase improves the G4 DNA unwinding, facilitates the DNA replication and the telomere elongation.

It has been proved by omics and large sample population studies that telomere length gradually decreases with human aging, and interacts with other important aging markers (such as stem cell function, mitochondrial function, and immune function), leading to a gradual decline in tissue renewal ability and inflammation. Telomere length has become one of the gold standards for detecting the process of aging and its related diseases [18-20].

To explore the full image of the molecular pathways that Bazhen decoction regulates, in this study, we adopted the RNA sequencing technique to dissect the molecular pathways regulated by Bazhen decoction. RNA sequencing could reveal all the RNAs that expressed under certain circumstance, thus provide the full image of transcriptome, in this case, the cellular transcriptome induced by Bazhen decoction treatment. By further analysis of the transcriptome, we found the DNA replication and telomere maintenance function been improved by Bazhen decoction treatment. To verify these data, we utilize the mouse embryo fibroblasts (MEFs) generated from Werner syndrome mouse model. Werner syndrome (WS) is an autosomal recessive genetic disease caused by DNA helicase Wrn gene mutation and telomere dysfunction. WS is characterized by premature aging and shortened life span. The average lifespan of WS is only 46-48 years, the symptoms include premature atherosclerosis, osteoporosis, cataracts, reproductive deficiency, type 2 diabetes mellitus, and soft-tissue sarcoma. We revealed that the Bazhen decoction elevated the expression of DNA helicases, which promoted the G4 DNA resolving, facilitated the DNA replication and the telomere elongation, and facilitate the progeroid Werner syndrome cell proliferation.

Results

The cellular transcriptome profile induced by application of Bazhen decoction revealed its systematic function in anti-aging

To get the full image of the molecular pathways regulated by Bazhen decoction, we applied the RNA sequencing (RNA-seq) technique to the cells with or without Bazhen decoction treatment. Due to the fact that many cell lines carry multiple gene mutations during the immortalization process, which might alter the response of affected molecular pathways to the Bazhen decoction, we utilized the primary cultured wild type mouse embryonic fibroblasts (WT MEF) to obtain the cellular transcriptome induced by Bazhen decoction.

After treatment of low dose (10μg/mL, BZT-L,1-3) and high dose (100μg/mL, BZT-H,1-3) of Bazhen decoction, the WT MEFs were applied for RNA sequencing to get the information of all the genes' mRNA count (expression level). The RNA-seq data was normalized and analyzed by single sample gene set enrichment analysis (ssGSEA) to get the cellular pathways activated (up-regulated) or inhibited (down-regulated) by Bazhen decoction.

The heatmap of top 50 pathways regulated by Bazhen decoction were plotted (Figure 1). Among the top up-regulated pathways, we found the stem cell pathway (Figure 1, BOQUEST STEM CELL), and Wnt-B-Catenin pathway which known to be important in stem cell regulation (Figure 1, WP REGULATION OF WNTBCATENIN SIGNALING BY SMALL MOLECULE COMPOUNDS). We also found the nicotinamide metabolism pathway (Figure 1, KEGG NICOTINATE AND NICOTINAMIDE METABOLISM), which is known to be essential for Sirtuins protein function and mitochondria function [21], was activated by Bazhen decoction (Figure 1).

Interestingly, our data revealed multiple pathways related to the protein glycosylation protein quality control and ER function were up-regulated by Bazhen decoction, such as glycosylphosphatidyl inositol (GPI) anchored protein synthesis (Figure 1, REACTOME POST TRANSLATIONAL MODIFICATION SYNTHESIS OF GPI ANCHORED PROTEINS), protein glycosylation (Figure 1, KEGG N GLYCAN BIOSYNTHESIS, REACTOME BASIGIN INTERACTIONS), the ER associated protein degradation pathway (Figure 1, BIOCARTA ERAD PATHWAY), the chaperon for unfolded proteins (Figure 1, REACTOME CALNEXIN CALRETICULIN CYCLE). These pathways are essential for maintaining protein homeostasis, reducing ER

stress and cellular apoptosis. The down-regulation of protein homeostasis has become one of the aging hallmarks [11].

It is very interesting that we found the neuronal function related pathways were up-regulated by Bazhen decoction, including the process of synaptic vesicle endocytosis (Figure 1, BIOCARTA NDKDYNAMIN PATHWAY), the sensation function (Figure 1, KEGG OLFACTORY TRANSDUCTION, REACTOME VISUAL PHOTOTRANSDUCTION). The down-regulation of these pathways are known to be the symptoms for neuron degenerative diseases.

Consistent with one of the original function (Nourishing hematopoietic system) for Bazhen decoction, we found the VEGFR3 signaling in lymphatic endothelium pathway (Figure 1, PID LYMPH ANGIOGENESIS PATHWAY) was activated, and the hemoglobin degradation pathway (Figure 1, REACTOME HEME DEGRADATION) was down-regulated. Other than this, the angiotensin converting and aldosterone synthesis inhibitor pathway (Figure 1, WP ACE INHIBITOR PATHWAY) was also up-regulated, which might help enhancing the wellness of cardiovascular system. To add on this finding, we also found that the transcriptional response to SARS-COV-2 (Figure 1, BLANCO MELO COVID19 BRONCHIAL EPITHELIAL CELLS SARS COV 2 INFECTION) has been down-regulated. Since the ACE2 is found to be the receptor for SARS-COV-2, these data suggest that the Bazhen decoction could block the SARS-COV-2 by affecting ACE proteins.

Among those down-regulated pathways, multiple inflammatory response pathways were found, including BIOCARTA TNFR2 PATHWAY, WP CORTICOTROPINRELEASING HORMONE SIGNALING PATHWAY, WP RESISTIN AS A REGULATOR OF INFLAMMATION, WP IL4 SIGNALING PATHWAY, PID L2 PATHWAY, BIOCARTA IL3 PATHWAY, REACTOME REGULATION OF TNFR1 SIGNALING, etc (Figure 1). Interestingly, the MAPK related signaling pathways were also down-regulated, including PID FCER1 PATHWAY, BIOCARTA TPO PATHWAY, WP GALANIN RECEPTOR PATHWAY, REACTOME FCERI MEDIATED MAPK ACTIVATION, PID FCER1 PATHWAY, WP HOSTPATHOGEN INTERACTION OF HUMAN CORONA VIRUSES MAPK SIGNALING etc. (Figure 1). MAPK related pathways are known to induce inflammatory response to stress, such as DNA damages, hypoxia, oxidative stress, virus infection, etc. The activation of MAPK pathways is the kind of double edged sword event. Consistent with this, the oxidative stress pathways were also down-regulated (Figure 1, WP OXIDATIVE STRESS, WEIGEL OXIDATIVE STRESS RESPONSE).

Although we observed the p53 regulated cell cycle arrest and caspase pathways (Figure 1, PID CASPASE PATHWAY, REACTOME TP53 REGULATES TRANSCRIPTION OF CASPASE ACTIVATORS AND CASPASES, REACTOME REGULATION OF TP53 ACTIVITY THROUGH ASSOCIATION WITH COFACTORS, REACTOME TP53 REGULATES TRANSCRIPTION OF GENES INVOLVED IN G1 CELL CYCLE ARREST, PID P73PATHWAY) were down-regulated by low dose of Bazhen decoction, we also observed the down-regulated of several tumorigenesis pathways, such as KEGG CHRONIC MYELOID LEUKEMIA, WP ENDOMETRIAL CANCER, WP PANCREATIC ADENOCARCINOMA PATHWAY, etc. (Figure 1).

Surprisingly, the target of rapamycin (TOR) signaling was also down-regulated (Figure 1, WP TARGET OF RAPAMYCIN TOR SIGNALING, PID TCR RAS PATHWAY), the inhibition of which is widely applied in anti-aging and anti-tumor drug screening.

Together these data revealed that the anti-aging function of Bazhen decoction might be achieved through systematic regulation of multiple anti-aging pathways, including stem cell regulation, maintaining protein homeostasis, promoting cardiovascular function, improving neuronal function, anti-inflammation, anti-DNA damage induced stress, etc.

The application of Bazhen decoction activated the telomere maintenance pathways *via* up-regulating the DNA helicases and telomere proteins

As we know, telomere function could be the sustaining mechanism for the stem cell function, anti-

inflammation, anti-DNA damage induced stress, etc., however, due to the low abundance of the genes in telomere maintenance pathway, it might not be able to stand out as top pathway in the transcriptome. Thus, we further plotted the heatmap for telomere function and DNA damage response related pathways and tried to dissect the function of Bazhen decoction in telomere maintenance.

Interestingly, we found that the telomere end processing related pathways, which is essential for telomere elongation and maintenance, were up-regulated (Figure 2, A, GOBP TELOMERE CAPPING, REACTOME POLYMERASE SWITCHING ON THE C STRAND OF THE TELOMERE, REACTOME TELOMERE C STRAND LAGGING STRAND SYNTHESIS, REACTOME TELOMERE C STRAND SYNTHESIS INITIATION, etc.). And the telomere DNA repair pathway was also up-regulated (Figure 2, A, GOBP TELOMERE MAINTENANCE IN RESPONSE TO DNA DAMAGE), which inhibited the telomere DNA damage induced cellular senescence (Figure 2, A, REACTOME DNA DAMAGE TELOMERE STRESS INDUCED SENESCENCE). We also found the up-regulation of DNA helicase pathways induced by Bazhen decoction (Figure 2, A, GOBP REGULATION OF HELICASE ACTIVITY, GOMF 3 5 DNA HELICASE ACTIVITY, GOMF DNA HELICASE ACTIVITY, GOMF HELICASE ACTIVITY, GOMF SINGLE STRANDED DNA HELICASE ACTIVITY). Interestingly, the telomerase pathway was not obviously regulated by Bazhen decoction (Figure 2, A, REACTOME TELOMERE EXTENSION BY TELOMERASE). We suspected that the telomere maintenance was through the activation of telomere recombination (Figure 2, A, up-regulation of GOBP TELOMERE MAINTENANCE VIA RECOMBINATION, and down-regulation of REACTOME INHIBITION OF DNA RECOMBINATION AT TELOMERE). The score of BZT-L1 was strangely high for some telomere and helicase pathways, but did not impact the tendency of up-regulation of these pathways by Bazhen decoction.

Consistent with this, the DNA damage repair pathways were also up-regulated (Figure 2, A, GOMF DAMAGED DNA BINDING, GOBP DNA SYNTHESIS INVOLVED IN DNA REPAIR, WP DNA REPAIR PATHWAYS FULL NETWORK, KAUFFMANN DNA REPAIR GENES). While the p53 regulated cell cycle arrest pathways were down-regulated by Bazhen decoction (Figure 2, A, REACTOME TP53 REGULATES TRANSCRIPTION OF GENES INVOLVED IN G1 CELL CYCLE ARREST, REACTOME TP53 REGULATES TRANSCRIPTION OF GENES INVOLVED IN G2 CELL CYCLE ARREST, REACTOME TP53 REGULATES TRANSCRIPTION OF CELL CYCLE GENES).

Together these data suggest that Bazhen decoction could promote DNA damage repair and telomere maintenance, reduce DNA replication stress, thus promote cell cycle progression, and prevent cellular senescence.

To verify these data and explore the role of Bazhen decoction in DNA replication and telomere maintenance, we utilized the progeroid MEF cells from Werner syndrome mouse (G3DKO cells, both DNA helicase Wrn and telomerase Terc genes were knocked out), with WT MEF cells as control. From the RNA-seq data, we realized that the low dose (10μg/mL) of Bazhen decoction showed better effect in sustaining cell growth, thus we treated the cells with 10μg/mL, and 20μg/mL Bazhen decoction for further experiments. The Western blot data showed that Bazhen decoction treatment up-regulated the protein level of DNA helicase Blm, Mcm7, Parp1 in both WT (Figure 2, B) and G3DKO cells (Figure 2, C), which are known to unwind DNA G4 structure, and promote DNA replication fork progression, especially in telomere DNA. The telomere protein Terf1, which is known to involve in telomere packaging and c-strand (lagging strand) synthesis, was also up-regulated. While the Pot1, which is known to involve in the negative regulation of telomere length, was down-regulated (Figure 2, B, C). Together these data suggest that the Bazhen decoction might regulate the telomere DNA replication and telomere DNA damage response, and thus regulate telomere lengthening.

At the same time, we also observed that the Bazhen decoction did not obviously affect the tumor suppressor p53 protein level, while the p53 down-stream effector p21 was up-regulated in both WT and G3DKO cells (Figure 2, C, D). Interestingly, another important tumor suppressor Rb family member Rb and p130, which known to be the cell cycle inhibitors, were up-regulated by Bazhen decoction. However, Rb pathway down-stream E2F1 was also up-regulated, which supposes to be the cell cycle promoter. These data suggest a double-side regulation of cell cycle progression by Bazhen decoction (Figure 2, C, D).

Together the above data suggest that Bazhen decoction could up-regulate the expression of DNA helicase, promote the unwinding of G4 structure, thus promote DNA replication, and regulate cell cycle progression.

The Bazhen decoction reduced the presence of G4 structure and endowed the cellular resistance to DNA replication stress induced by G4 stabilizer

We further investigate the impact of Bazhen decoction on G4 structure, DNA replication stress induced DNA damage, cell cycle progression. We first used anti-G4 antibody to detect the amount of G4 structure in G3DKO cells before and after Bazhen decoction treatment. We found that the signals for G4 structure were obviously diminished after Bazhen decoction treatment (Figure 3, A, B, G4), and the signals for DNA helicase Recql4 (Figure 3, A, Recql4) and Blm (Figure 3, B, Blm) were enhanced, and colocalized with G4 structure. These data suggest that the Bazhen decoction promote the resolving of G4 structure by facilitating the recruitment of DNA helicases Recql4 and Blm to G4 DNA. To further verify these data, we applied the G4 stabilizer pyridostatin (PDS) to stabilize G4 structure and induce DNA replication stress (DNA damage). As expected, comparing with control cells, the PDS treatment stabilized and increased the amount of G4 structure detected, while the Bazhen decoction could obviously diminish the G4 signals stabilized by PDS (Figure 3, A, B, G4).

We also revealed that the PDS treatment could down-regulate the DNA helicase Blm, Recql4, Parp1, Mcm7, telomere protein Terf1, and cell cycle protein Cdc2, Cdk2 in G3DKO cells, while Bazhen decoction could rescue the expression of these proteins (Figure 4, A). The cell cycle analysis revealed that Bazhen decoction could promote the cell cycle progression, reduced the percentage of G1 phase cells, and increase the percentage of S phase and G2 phase (Figure 4, B). The PDS treatment also induced the increase of G2 cells, which might due to the DNA damage repairing events induced by DNA replication stress. The Bazhen decoction application further increased the G2 phase cells, which might ensure the proper repair of DNA damage induced by PDS. To verify this, we detect the DNA damages induced by PDS with/without Bazhen decoction. We could observe the signal of DNA damage marker γ-H2AX in the G3DKO cells without any treatment (Figure 4, C, control), this might due to the background DNA replication stress induced by Wrn and telomerase knock out. The application of Bazhen decoction obviously reduced theγ-H2AX signal, indicates that the DNA damages induced in premature aging cells were repaired (Figure 4, C, BZT). Compared to these, the cells treated with PDS showed increasedγ-H2AX signals, indicated the induction of DNA damages by PDS (Figure 4, C, PDS). While the application of Bazhen decoction obviously diminished theγ-H2AX signals induced by PDS, indicates that Bazhen decoction could facilitate the repair of DNA damages induced by G4 stabilization (DNA replication stress) (Figure 4, C, PDS+BZT).

Together these data suggest that the Bazhen decoction could promote the resolving of G4 structure and endow the cellular resistance to PDS induced DNA replication stress, thus enhance the quality control of cell cycle.

The Bazhen decoction facilitated the telomere elongation

The above data indicate that Bazhen decoction could facilitate the resolving of G4 structure, which is known to be abundant structure in telomere DNA, thus Bazhen decoction might facilitate telomere DNA replication and elongation. To verify this, we performed real-time PCR to compare the relative amount of telomere DNA before and after Bazhen decoction treatment. The results showed that the telomere DNA increased significantly after Bazhen decoction treatment ($p<0.01$) (Figure 5, A). We further performed immunofluorescence together with fluorescence *in situ* hybridization (IF-FISH) to detect the recruitment of DNA helicase to telomere DNA. Consistent with the real-time PCR data, IF-FISH data showed that the DNA helicase Recql4 and Blm were up-regulated (Figure 5, B, C, Recql4, Blm), and the telomere DNA signals significantly increased after Bazhen decoction treatment (Figure 5, B, C, Telomere), the colocalization of DNA helicase Recql4 with telomere DNA increased (Figure 5, B, C, Telomere & Recql4, white arrows), same with the Blm (Figure 5, B, C, Telomere & Blm, white arrows). These data suggest that Bazhen decoction up-regulated DNA helicases and also facilitated the recruitment of DNA helicases to the telomere DNA, which might promote G4 structure resolving and telomere elongation.

Discussion

The classical TCM prescriptions, such as Bazhen decoction, have been applied clinically and showing effective in treatment of aging-related degenerative diseases or for longevity sustaining function. However, due to the limit in the pharmacological study of such complicated TCM system, it is hard to evaluate the pharmacological effect and follow up the clinical outcome, especially for aging related degenerative diseases.

Here we try to apply a systematic pharmacological methodology to study classical TCM prescriptions. Through RNA sequencing and ssGSEA analysis, we obtained the transcriptome profile regulated by Bazhen decoction in a wild type genetic background. This way we could avoid the alteration of signaling pathways caused by gene mutations occurred during the cell line establishment process. The transcriptome profile of wild type cells regulated Bazhen decoction revealed a systematic regulation of multiple anti-aging pathways, including stem cell regulation, protein homeostasis, cardiovascular function, neuronal function, anti-inflammation, anti-DNA damage induced stress, etc. by Bazhen decoction (Figure 1). This transcriptome profile and detailed signaling pathways will be very useful for further study of Bazhen decoction's interfering function in various organ degenerative diseases.

For the above possible anti-aging properties, telomere maintenance could be a common molecular basis for sustaining the stem cell function, preventing DNA damage, improving organ function, anti-inflammation, etc. Consistent with this, our further analysis of the transcriptome revealed that Bazhen decoction treatment could up-regulate the pathways for DNA helicase activity and telomere lengthening (Figure 2, A).

It is well known that the smooth progression of DNA replication is essential for telomere maintenance and cell cycle progression. However, the DNA secondary structure, such as telomere G-quadruplex (G4) structure, slows down the replication fork progression, cause replication stress, and become the chromosome fragile sites [22]. The function of DNA helicase is to fire replication origins and unwind the G4 structures, ensure the accurate formation of replication forks and the smooth progression of DNA replication. The defect of helicase function result in DNA replication stress, activates DNA damage response pathways, and results in aging or tumorigenesis [16, 23]. The genetic defect of helicase causes progeroid syndromes, such as Werner syndrome (Wrn gene mutation) [24], Bloom syndrome (Blm gene mutation) [25], Rothmund-Thomson syndrome (Recql4 gene mutation) [26], etc.

To further understand the regulation of Bazhen decoction on DNA helicases and telomere, with the progeroid cells derived from Werner syndrome mice, we verified that DNA helicases Blm, Recql4, Parp1, Mcm7 and telomere regulating protein Terf1 were up-regulated by Bazhen decoction, and Pot1 were down-regulated (Figure 2, B-E). Studies have shown that helicase Blm has a strong activity for unwinding G4 structures and is important for the synthesis of telomeric DNA leading strand [27]. Helicase Fancm, Brca1, and Blm regulate DNA end processing and homologous recombination [28]. Other than this, the Blm protein could resolve other secondary DNA structure, such as Holiday junction, thus is important for the regulation of DNA replication stress. The Recql4 protein is involved in the repair of DNA double strand breaks, as well as the telomere DNA maintenance [29]. Thus the up-regulated DNA helicases by Bazhen decoction promote the resolving of G4 structure, and endowed the cellular resistance to DNA damages induced by G4 stabilizer PDS, promoted the quality control of cell cycle and telomere elongation (Figure 3-5).

Together these data suggest that Bazhen decoction facilitate G4 resolving and telomere maintenance, which might contribute to the longevity sustaining properties revealed by transcriptome profile. Thus the Bazhen decoction application could be a strategy of dealing with DNA replication stress, and the prevention of the progress of aging, as well as tumorigenesis.

Our data also suggest a possibility of Bazhen decoction in treating genetic progeroid syndromes, especially those with telomere dysfunction. Indeed, some physician have used Bazhen decoction to treat aplastic anemia [30, 31]. The hereditary aplastic anemia is due to abnormal telomerase (e.g., dyskeratosis congenica syndrome) or DNA helicase (e.g., Fanconi's anemia) function [32, 33]. While the cause of sporadic aplastic anemia is mainly the DNA damage of hematopoietic stem cells. Further study on Werner syndrome mouse model will be performed to evaluate the function of Bazhen decoction on organ aging and related diseases.

Surprisingly, from the transcriptome profile, we also revealed that Bazhen decoction could be applied in prevention and treatment of COVID-19 (Figure 1). Interestingly, the COVID-19 has known to damage the digestion function and cardiovascular function, which is the two targets for Bazhen decoction (The Sijunzi decoction protect the digestion function, and the Siwu decoction protect the cardiovascular function). The anti-inflammation profile might also contribute to the COVID-19 treatment.

Together, our data provide the full image of possible pathways regulated by Bazhen decoction. The signature pathways revealed by transcriptome analysis are more accurate and stable than individual gene variation, could applied as biomarkers for TCM system. With further clinical studies, these key signaling pathways of TCM prescription could be applied in quantification evaluation of clinical pharmacological effect to a certain extent, and explain the role and molecular mechanism of classic longevity formulae in the prevention and treatment of aging-related diseases, so as to guide the clinical precision treatment of traditional Chinese medicine.

Materials and Methods

The preparation of Bazhen decoction

The formula of Bazhen decoction is as the following: Panax ginseng 30g, Radix rehmanniae praeparata 30g, Radix paeoniae alba 30g, Ligusticum wallichii 30g, Poria cocos 30g, Angelica sinensis 30g, Rhizoma Atractylodis Macrocephalae 30g, Licorice 30g. The herbs were immersed in 800 mL distilled water, soaked for 3 hours, and boiled twice to get the water extract solution. The extracts were combined, filtered, and concentrated to 0.6 g/mL. The final solution was sonicated and centrifuged (3000rmp, 15min), the supernatant was collected, filtered with 0.45μM membrane, aliquot, and stored in -20°C.

Cell culture and treatment

All mouse protocols have been approved by the Animal Care and Use Committee of Guizhou Medical University. The MEF cells of WT (wild type) and G3DKO (G3$mTR^{-/-}mTerc^{-/-}$) were harvested in e13.5 days and cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) at 37 with 5% CO_2 and 3% O_2. The MEFs were used for experiments before passage 5 and the cells were passaged at a ratio of 1:2 or 1:3.

The cultured cells were treated with Bazhen decoction (control, low dose (10μg/mL), high dose (20μg/mL)) for 48hr. For G4 structure stabilizer treatment, the cells were treated with 5μM pyridostatin hydrochloride (PDS) (MCE) for 24h. For combined treatment of Bazhen decoction and PDS, the cells were treated with Bazhen decoction (control, low dose (10μg/mL), high dose (20μg/mL)) for 24hr, then add 5μM PDS and continue the treatment for another 24hr.

RNA sequencing (RNA-seq) and transcriptome data analysis

The WT cells were treated with Bazhen decoction (control, low dose (10μg/mL), high dose (100μg/mL)) for 24hr. The cells were harvested and sent to perform RNA-seq by commercial service. For each sample, 20G of RNA-seq data were collected for further analysis.

The single sample gene set enrichment analysis (ssGSEA) [34] was performed to analyze the RNA-seq to get the scores for signaling pathways. The C2 and C5 gene sets from Molecular Signatures database [35] were used as the pathway database for ssGSEA analysis. The top differential regulated pathways were plotted with Morpheus (https://software.broadinstitute.org/morpheus) to obtain the transcriptome profile regulated by Bazhen decoction.

Western blot

Cells were harvested and lysed in RIPA buffer containing protease inhibitor cocktail (Roche, Switzerland). The 20 μg of total protein were separated by SDS-PAGE and then transferred to PVDF membrane. After blocking in 10% non-fat milk for 1 h at room temperature, membranes were incubated with primary antibodies overnight at 4°C. The membranes were then incubated with horseradish peroxidase-labeled seconda-

ry antibodies, and visualized with ECL. The primary antibodies used were anti-Blm (1:1000, Invitrogen), anti-Recql4 (1:1000, Invitrogen), anti-Pot1 (1:1000, Invitrogen), anti-Terf1 (1:1000, Invitrogen), anti-Mcm7 (1:1000, Santa Cruz), anti-Parp1 (1:1000, Abcam), anti-p53 (1:5000, Proteintech), anti-Rb (1:1000, BD pharmingen), anti-p130 (1:1000, Abcam), anti-E2F1 (1:1000, Novus), anti-Cdc2 (1:1000, CST), anti-Cdk2 (1:1000, CST), anti-actin (1:1000, Santa Cruz).

Flow cytometry assay

For cell cycle analysis, cells treated with or without PDS were harvested and fixed in ice-cold 70% ethanol, stained with 1X PBS based propidium iodide solution (50μg/mL PI, 100μg/mL RNase A, 0.1% sodium citrate, 0.1% Triton X-100), and analyzed by flow cytometry (Agilent).

Immunofluorescence

The cells cultured on cover slips were fixed with 2% paraformaldehyde and 2% sucrose in 1×PBS for 10 min and then permeabilized with 1% NP-40. After pre-incubation with 5% BSA/PBS, cells were incubated first with the primary antibody and then with the secondary antibody in 1% BSA/PBS for 1 h at room temperature. The slides were mounted with anti-fade mounting medium with DAPI (Solarbio). The primary antibodies used were anti-γH2AX (1:1000, Abcam), anti-DNA G-quadruplex (G4) antibody, clone 1H6 (1:200, Merck), anti-Blm (1:200, Invitrogen), anti-Recql4 (1:200, Invitrogen).

Telomere assays

Immunofluorescence and fluorescence in situ hybridization (IF-FISH): Cells cultured on coverslips were treated with Bazhen decoction (control, low dose (10μg/mL), high dose (20μg/mL)) for 48 hours. After washing three times in 1×PBS, cells were fixed for 10 minutes in 2% sucrose and 2% paraformaldehyde, and permeabilized with 0.5% NP-40. After blocking in blocking solution (2% Gelatin and 0.5% BSA in 1xPBS), the primary antibody were applied overnight at 4degC and followed by secondary antibody incubation for 1h at room temperature in the dark. After washing three times in 1xPBST, the cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature, and hybridized with a telomere PNA-FISH probe 5'-FITC-green-(TTAGGG)-3' (Panagene). Coverslips were washed and counterstained with anti-fade mounting medium with DAPI (Solarbio). The primary antibodies for IF-FISH: anti-Recql4 (1:200, Invitrogen), anti-Blm (1:200, Invitrogen).

Telomere DNA qPCR assay: The cultured cells (1x10^{5}cells/well in 6-well plate) were treated with Bazhen decoction (control, low dose (10μg/mL), high dose (20μg/mL)) for 48hr. Then the cells were passaged for six times (1x10^5 cells/well in 6-well plate) and continued with the Bazhen decoction treatment. The 7th passage cells were harvested and the genomic DNA was purified for Syber green based real time PCR. The telomere DNA primer sequences were: Forward: 5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3', Reverse: 5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'. The reference 36B4 DNA primer sequences were: Forward: 5'-ACTGGTCTAGGACCCGAGAAG-3', Reverse: 5'-TCAATGGTGCCTCTGGAGATT-3'.

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Declaration of interest

The authors declare no conflict of interest.

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Figure Legends:

Figure 1. The top 50 signaling pathways regulated by Bazhen decoction.

BZT C1-C3 were three control samples, BZT L1-L3 were three samples with low dose (10μg/mL) Bazhen decoction treatment, BZT H1-H3 were three samples with high dose (100μg/mL) Bazhen decoction treatment. The red color stands for up-regulation, the blue color stands for down-regulation.

Figure 2. The Bazhen decoction activated the DNA helicase and telomere maintenance pathways. A. The DNA helicase and telomere maintenance pathways were up-regulated by Bazhen decoction. B-C. After Bazhen decoction treatment, the protein level of DNA helicase Blm, Mcm7, Parp1 and telomere protein Terf1were up-regulated, while telomere protein Pot1 was down-regulated in WT cells (B) or G3DKO cells (C).

D-E. After Bazhen decoction treatment, the protein level of cell cycle inhibitor p21, Rb, and p130 were up-regulated, while cell cycle promoter E2F1 was also up-regulated in WT cells (D) or G3DKO cells (E).

Figure 3. In G3DKO cells, the Bazhen decoction up-regulated the DNA helicase, promoted the resolving of G4 structure.

The signals of G4 structure were diminished by Bazhen decoction, while the Recql4 signals were elevated. The PDS treatment stabilized the G4 signals, and down-regulated the Recql4, while combined Bazhen decoction rescued the Recql4 expression and diminished the G4 signals.

The signals of G4 structure were diminished by Bazhen decoction, while the Blm signals were elevated. The PDS treatment stabilized the G4 signals, and down-regulated the Blm, while combined Bazhen decoction rescued the Blm expression and diminished the G4 signals.

Figure 4. In G3DKO cells, the Bazhen decoction regulated cell cycle progression, and protected the cells from DNA damages induced by G4 stabilizer PDS.

1. In G3DKO cells, PDS treatment down-regulate the DNA helicase Blm, Recql4, Parp1, Mcm7, telomere protein Terf1, and cell cycle protein Cdc2, Cdk2, while Bazhen decoction rescued the expression of these proteins.
2. The flow cytometry analysis of G3DKO cells showed that the percentage of G1 phase cells was reduced and the percentage of S phase and G2 phase was increased. The PDS treatment also induced the increase of G2 cells.
3. The Bazhen decoction application reduced the signal of DNA damage marker γ-H2AX in the G3DKO cells. The cells treated with PDS showed increasedγ-H2AX signals, while the application of Bazhen decoction obviously diminished theγ-H2AX signals induced by PDS.

Figure 5. Bazhen decoction treatment facilitate the elongation of telomere.

1. Real-time PCR data showed that the telomere DNA increased significantly after Bazhen decoction treatment ($p<0.01$).
2. IF-FISH showed that the DNA helicase Recql4 was up-regulated (Recql4), and the telomere DNA signals significantly increased after Bazhen decoction treatment (Telomere), the colocalization of Recql4 with telomere DNA increased (Telomere & Recql4, white arrows pointed to the colocalization spots).
3. IF-FISH showed that the DNA helicase Blm was up-regulated (Blm), and the telomere DNA signals significantly increased after Bazhen decoction treatment (Telomere), the colocalization of Blm with telomere DNA increased (Telomere & Blm, white arrows pointed to the colocalization spots).

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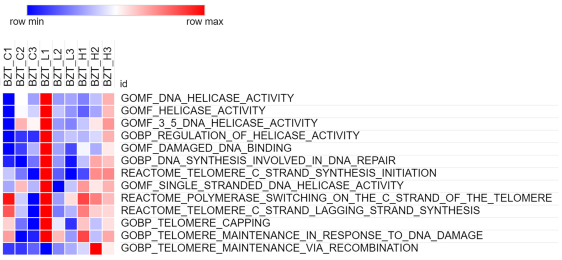

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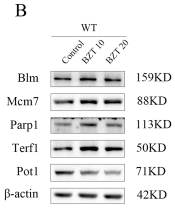

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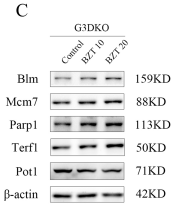

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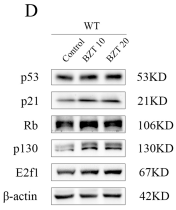

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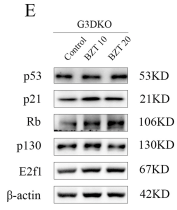

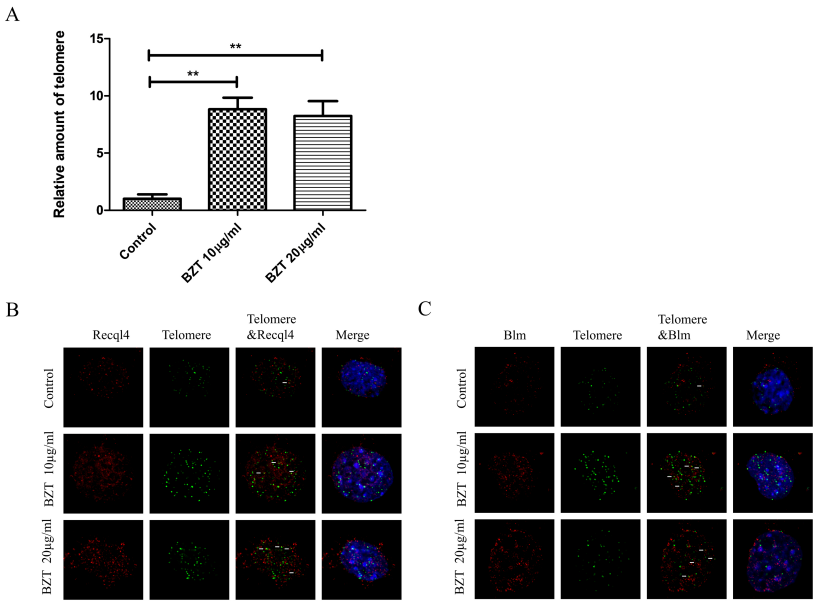

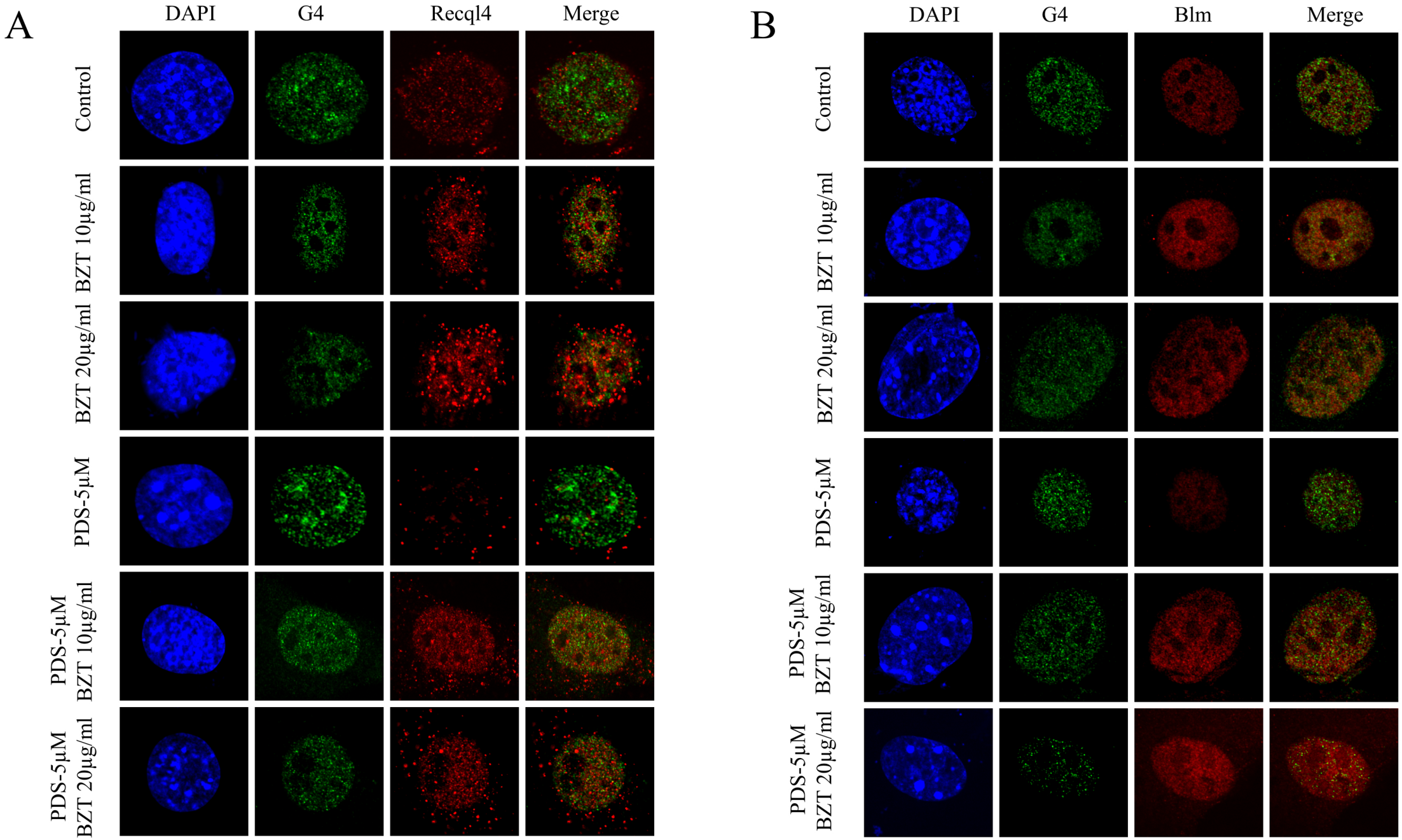
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Recql4
Merge
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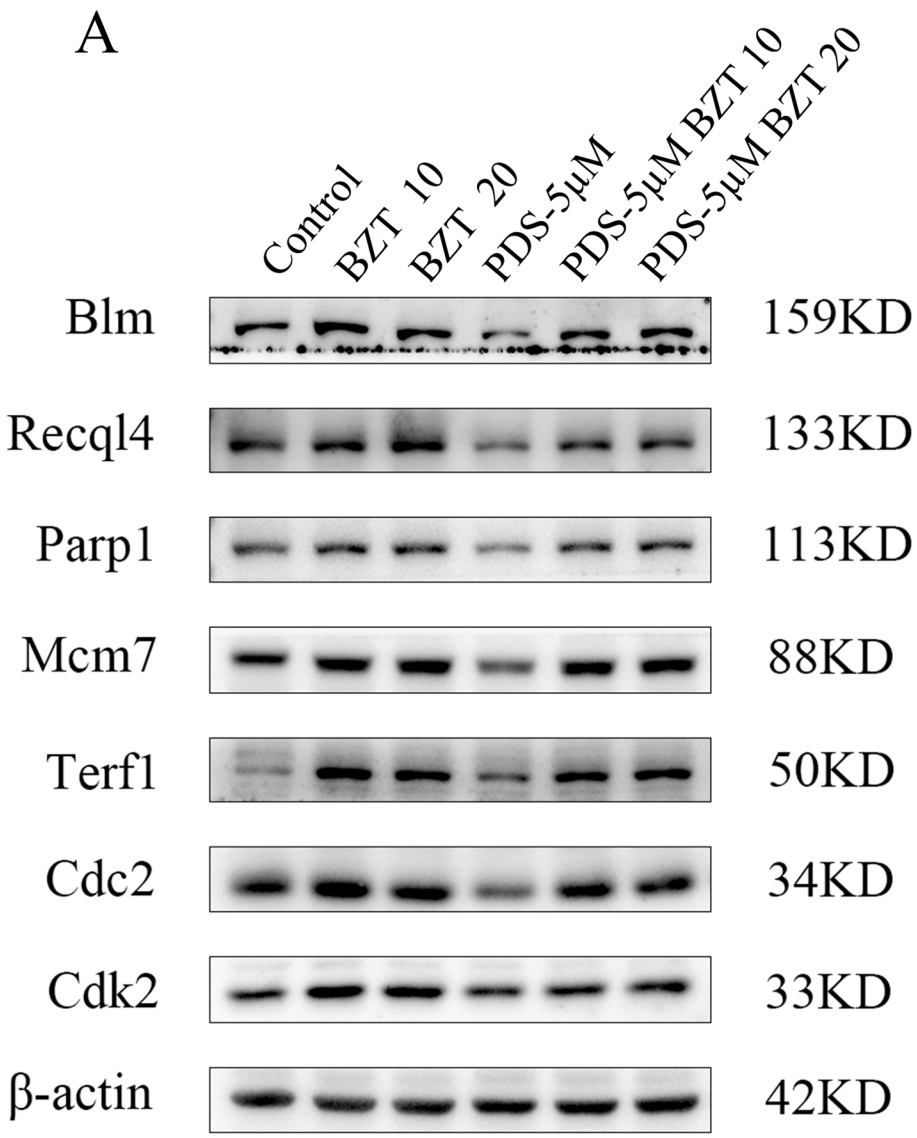
A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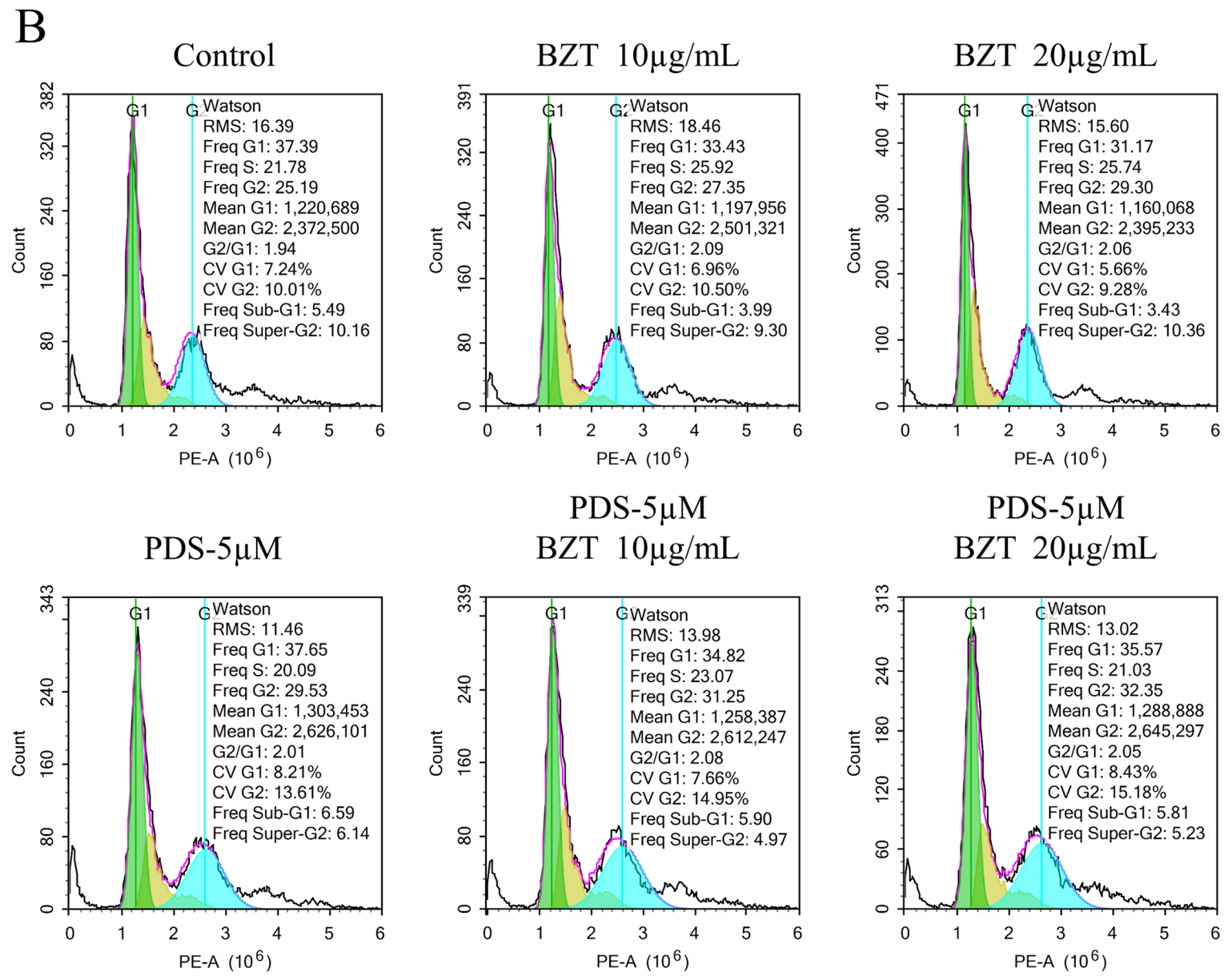
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Freq S: 21.78
Freq G2: 25.19
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Mean G2: 2,372,500
G2/G1: 1.94
CV G1: 7.24%
CV G2: 10.01%
Freq Sub-G1: 5.49
Freq Super-G2: 10.16
BZT 10μg/mL
Watson
RMS: 18.46
Freq G1: 33.43
Freq S: 25.92
Freq G2: 27.35
Mean G1: 1,197,956
Mean G2: 2,501,321
G2/G1: 2.09
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CV G2: 10.50%
Freq Sub-G1: 3.99
Freq Super-G2: 9.30
BZT 20μg/mL
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Mean G2: 2,395,233
G2/G1: 2.06
CV G1: 5.66%
CV G2: 9.28%
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Freq Super-G2: 10.36
PDS-5μM
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Freq S: 20.09
Freq G2: 29.53
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Mean G2: 2,626,101
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CV G1: 8.21%
CV G2: 13.61%
Freq Sub-G1: 6.59
Freq Super-G2: 6.14
PDS-5μM BZT 10μg/mL
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Freq G2: 32.35
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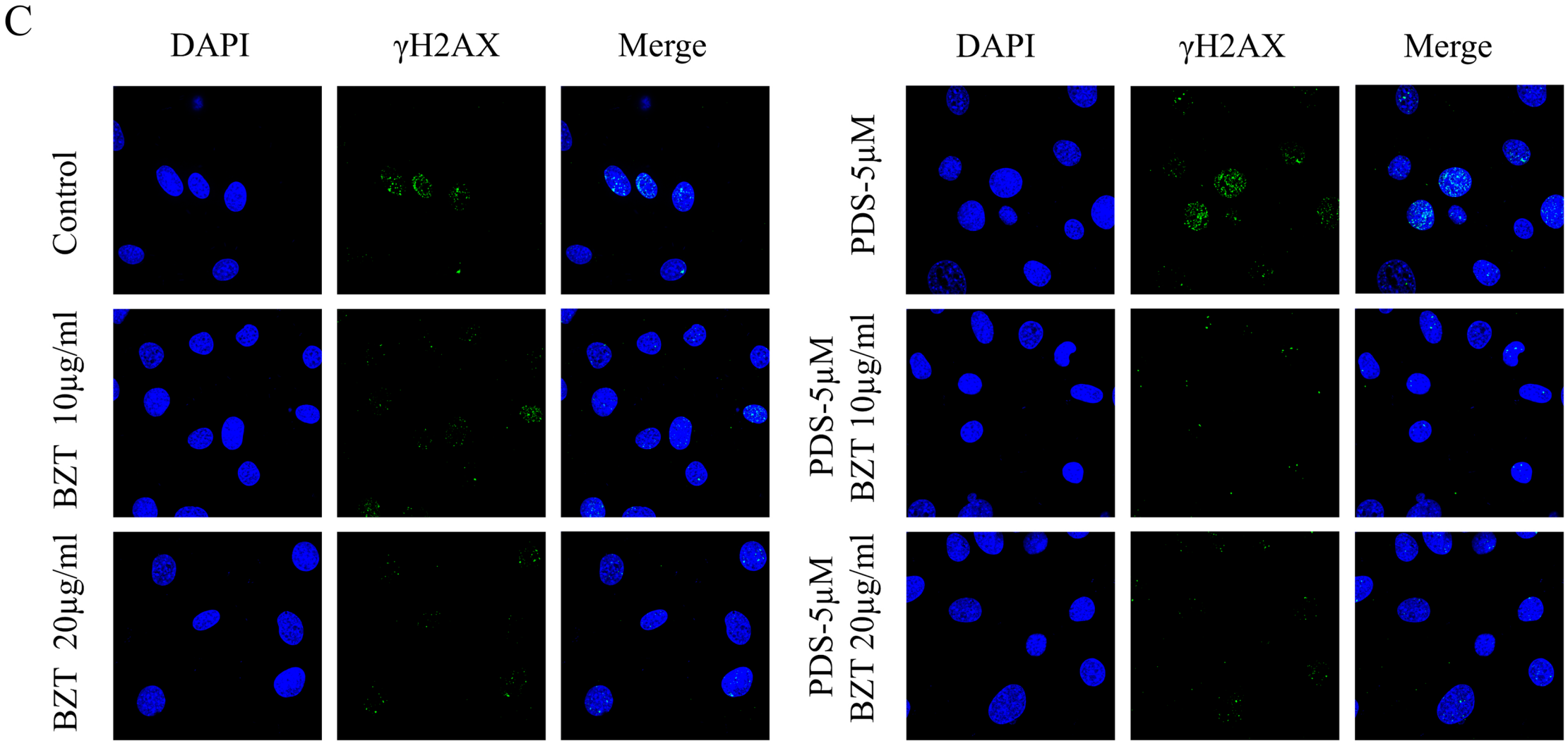
C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