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Master's Thesis

Baicalein, a small molecule regulating R-loop  
formation

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# Baicalein, a small molecule regulating R-loop formation

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Soomin Kim

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Approved by



Advisor

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# Baicalein, a small molecule regulating R-loop formation

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06/05/2019

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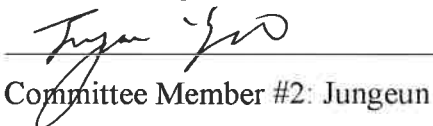
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## Abstract

R-loop is a tri-nucleotide structure composed of DNA:RNA hybrid and non-template ssDNA. Tight regulation of R-loop formation is important for maintaining genomic stability. Abnormal R-loop formations are shown in some cancers and neurological disorders. Some of small molecules commonly used as DNA damaging reagents are discovered to regulate R-loop formation. For example, camptothecin (CPT), which induces DNA damage by inhibiting Topoisomerase I, enhances R-loop formation. To find out small molecules regulating R-loop formation, High Content Screening (HCS) to visualize R-loops in high-throughput manner was established. Baicalein was identified as the first small molecule reducing CPT-induced R-loops. Though it is still unclear whether baicalein directly binds to R-loop, R-loop reduction by baicalein depended on XPF. DNA damage marker, 53BP1 recruitment to chromatin was decreased by baicalein treatment in XPF-dependent manner. However, the catalytic activity of XPF was dispensable for reduction of R-loop formation and 53BP1 recruitment to chromatin by baicalein treatment. Collectively, I found a small molecule, baicalein, can regulate R-loop formation and 53BP1 recruitment to the chromatin in XPF dependent manner.



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## **Abbreviations**

DSB : double strand break

HCS : high content screening

CPT : camptothecin

MMC : mitomycin C

HB-GFP : hybrid of DNA:RNA binding domain of RNASEH1 and EGFP

SRP : surface resonance plasmon

NER : nucleotide excision repair

GG-NER : global genomic nucleotide excision repair

TC-NER : transcription-coupled nucleotide excision repair

NHEJ : non-homologous end joining

HR : homologous recombination

ICL : interstrand cross-linking

dsDNA : double strand DNA

MMR : mismatch repair

PLA : Proximity ligation assay

## Introduction

R-loop is one of the non-B DNA structure composed of RNA/DNA hybrid and associated non-template single-stranded DNA. It is formed both in physiological and DNA damage condition. In the cellular process, R-loops are involved in the lagging-strand synthesis, DNA repair, regulation of gene expression, mitochondrial DNA replication, class-switch recombination in B cells and CpG-island promoter unmethylation. Due to the positive and negative effects of R-loop in cells, the formation of R-loop should be precisely regulated [1]. The disruption of this homeostasis can induce replication fork collapse and double strand breaks (DSBs) and potentially cause several neurological diseases and cancers [2, 3]. However, the exact molecular mechanism of R-loop resolution and the chemical that can regulate R-loop formation is not clearly understood since there are limited tools such as chemicals affecting R-loop formation. Therefore, I decided to find novel proteins and chemicals that affect R-loop formation to understand pathological mechanisms driven by R-loop formation and potentially identify therapeutic approaches for R-loop related diseases. Recently, many DNA repair proteins were identified to function in R-loop formation suggesting some DNA repair pathways actively regulates R-loop formation.

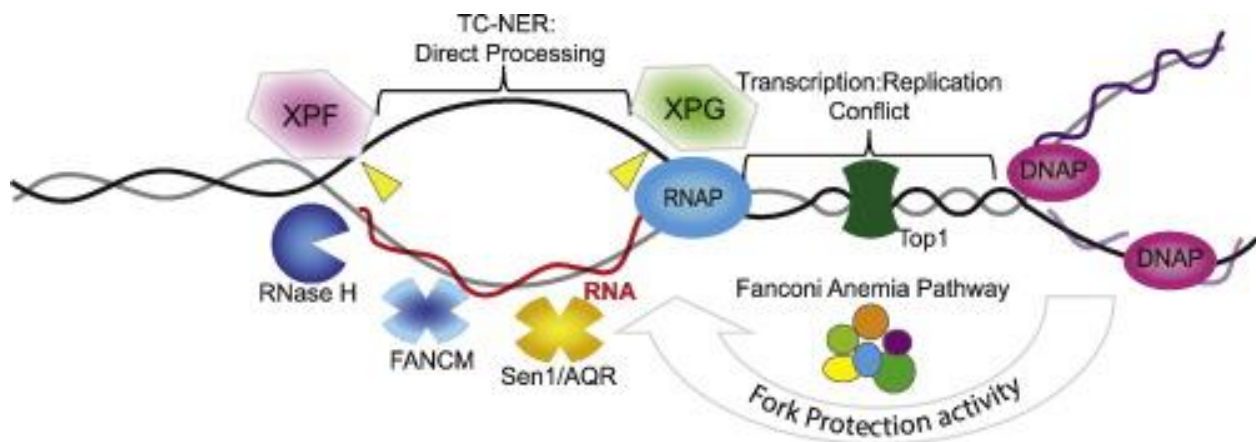
Many cancers are caused by some malfunctioning DNA repair mechanisms. Therefore, scientists are interested in linkage between DNA repair and cancer metabolism and search for small molecules targeting DNA repair [4]. For example, olaparib, which is a PARP1 inhibitor, is used for the treatment of BRCA-mutated ovarian cancer [5]. Baicalein (5, 6, 7-trihydroxy-2-phenyl-4H-1-benzopyran-4-one) is a flavone, which is a natural product extracted from the roots of *Scutellaria baicalensis* [6]. It affects diverse roles in biological activities such as a positive allosteric modulator of GABA<sub>A</sub> receptor [7], anti-cancer reagent [6], and antagonist of estrogen receptor [8]. In the recent study, the novel role of baicalein in DNA repair, especially in mismatch repair was revealed. Baicalein selectively kills mismatch repair deficient cells and binds preferentially with mismatched DNA and interacts with MSH2 and MSH6 proteins [9]. It has not been discovered whether baicalein can affect other bubble containing DNA structures.

Nucleotide excision repair (NER) removes bulky DNA lesions from genome. There are two sub pathways of NER; global genomic NER (GG-NER) and transcription-coupled NER (TC-NER). TC-NER resolve R-loop formation. (Figure 1, [10]). XPF is an 3' to 5' endonuclease of a NER lesion and functions to make a single strand break at 5' end whereas XPG makes a single strand break at 3' end of a bubble structure during TC-NER, respectively [11]. It has been reported that baicalein treatment induced  $\gamma$ -H2AX formation in XPF dependent manner in mismatch repair deficient cells [9]. The molecular mechanism of XPF and baicalein has not been revealed so far.

DSBs are mostly repaired by non-homologous end joining (NHEJ) repair and homologous recombination (HR). 53BP1 and BRCA1 drive NHEJ and HR, respectively [12, 13]. It has been

suggested that repair proteins in NER or mismatch repair (MMR) have a role in DSB repair [14, 15]. For example, XPF-ERCC1 complex is recruited by RAD52 on DSB and remove non-homologous 3' single strand tails in alternative end-joining [16]. However, the direct relationship between 53BP1 and XPF/ERCC1 is still unknown.

Here, I established screening methods using imaging and flow cytometry assay to find small molecules regulating R-loop. Among them, baicalein was found to decrease R-loop formation in the presence of XPF without affecting DSB efficiency. Also, my data exhibit a clue suggesting the relationship between XPF and 53BP1.



**Figure 1. Model of TC-NER (Transcription-coupled Nucleotide Excision repair) in transcription-replication conflict [10]**

## Materials and Methods

### 1. DNA sequences used in SPR experiment and EtBr displacement assay

Upstream DNA sequence : 5'-AGT GGT TCC CAT ATC CCG GAC GAG-3', Upstream RNA sequence : 5'-AGU GGU UCC CAU AUC CCG GAC GAG-3', Downstream DNA sequence : 5'-GGG ATC AGT GGT TCC CAT ATC CCG GAC GAG CCC CCA-3'

### 2. Antibodies

S9.6 (Kerafast, ENH001, mouse), XPF 3F2/3 (Santacruz, sc-136153, mouse), XPF (NOVUS, NB100-60679, rabbit), RNASEH1 (Invitrogen, PA5-30974, rabbit), TOPOI C-21 (Santacruz, sc-32736, mouse),  $\beta$ -ACTIN (Sigma, 105M-4866V, mouse), 53BP1 (Abcam, ab21083, rabbit), BRCA1 (Santacruz, sc-6954, mouse), H2AX S139 (Sigma Aldrich, 05636, mouse), H3, pan (upstate, 07-690, rabbit), pATM S1981 (Abcam, ab81292, rabbit), mouse monoclonal phosphor-Histone H2AX (Ser139) (Millipore, #05-636)

### 3. Plasmids

HB-GFP was a gift from Dr. Kwangseog Ahn [17].

### 4. Cell culture

5. XP2YO cell lines were gifts from Dr. Schärer OD [18]. HeLa, U2OS, and XP2YO cell lines were cultured in DMEM (HyClone™) with 1xPen-Sterp supplemented by 10% fetal bovine serum (FBS). All cell lines were cultured in humid incubator with 5% CO<sub>2</sub> at 37°C.

### 6. R-loop staining

R-loops was stained with S9.6 antibody. Cells were harvested and treated with hypotonic solution (75 mM KCl). Cells were then fixed on clean glass slides by methanol. After blocking for 1 hour with blocking solution (5% BSA and 0.5% Triton X-100), the fixed cells were subjected to stain with S9.6 antibody.

### 7. Immunofluorescence

Cells were seeded 24hours before the treatment of small molecules. Cells were then washed

with ice-cold PBS. Cells were permeabilized using cytoskeletal (CSK) buffer (10 mM PIPES (pH 6.8), 100 mM NaCl, 300 mM Sucrose, 3 mM MgCl<sub>2</sub>, 1 mM EGTA (pH 7.5)) with 0.5% Triton X-100. For S9.6 antibody, 0.5% of Triton X-100 in PBS was used for permeabilization instead of CSK buffer. Cells were washed and fixed with 4% of paraformaldehyde for 20 minutes in dark condition and following incubation for blocking with 10% FBS at room temperature for 1 hour. The fixed cells were subjected to stain with indicated antibodies for over-night at 4°C. Cells were then incubated with fluorescence-conjugated second antibodies (Invitrogen) and mounted with permanent mounting medium with DAPI (Vector Labs).

#### 8. Chromatin fractionation

Soluble form of the cells was extracted using M-PER™ (Thermo Fisher) with 1X benzonase. The chromatin-bound fraction was isolated by resuspending the insoluble pellet by RIPA buffer (150 mM NaCl, 1 % NP-40 or Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris (pH 8.0), 10 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, and protease inhibitors (Roche)) on ice for 30 minutes followed by sonication and centrifugation.

#### 9. Western blot analysis

To extract whole proteins in cells, cells were lysed by M-PER (ThermoFisher) with phosphatase and protease inhibitor cocktail (ThermoFisher) at 4°C. The amount of proteins was quantified with Bradford assay (Bio-Rad). 4-20% of Invitrogen gradient gel and MES buffer were used for separation of proteins by their sizes. Proteins in gel were transferred to polyvinylidene difluoride (PVDF) membrane in Tris-glycine buffer (Bio-Rad) with 20% of methanol by electrical current. Chemidoc (Bio-Rad) was used to obtain Western blotting images.

#### 10. EtBr displacement assay

Fluorescence of EtBr was recorded in Synergy HTX Microplate Reader at excitation and emission wavelengths of 485nm and 612nm respectively. Each sample contained 100ng of either double strand DNA (dsDNA) or DNA:RNA hybrid with 6.43 μM of EtBr. 0.2 mM, 0.4 mM, 0.8 mM of netropsin, baicalein, or CPT was added in samples and calculated using the following equation.

$$\text{Percentage of relative fluorescence} = \frac{(F_{obs} - F_0) \times 100}{(F_{DNA} - F_0)}$$

$F_0$ ,  $F_{DNA}$ ,  $F_{obs}$  are the fluorescence intensities of unbound EtBr, EtBr intercalated with DNA and EtBr-DNA complex with small molecules respectively.

#### 11. Proximity ligation assay

PLA was done with Duolink<sup>®</sup> PLA fluorescence protocol (Sigma Aldrich). Cells were washed with PBS and incubated with CSK buffer at 4°C for 10 minutes. Cells were then fixed with 4% paraformaldehyde for 20 minutes at room temperature and blocked with 10% of PBS for an hour at 37°C. S9.6, XPF, and 53BP1 antibodies were incubated for overnight at 4°C. Three times wash with PLA wash buffer A was done before addition of Duolink<sup>®</sup> antibody diluent. Ligation, amplification and washing with wash buffer B were done as in the protocol. Cells were mounted with mounting solution and put on the coverslips.

#### 12. Colony forming assay

Cells were seeded after treatment of DNA damaging reagents. After 14 days of incubation, survived colonies were washed with PBS and stained with 2 ml of methylene blue for 5 minutes at RT. The plates were rinsed with tap water and air-dried for few days. Colonies were counted with a stereomicroscope and plating efficiency (PE) and surviving fraction (Sf) were calculated.

$$PE = \frac{(\text{number of colonies formed})}{(\text{number of cells seeded})} \times 100\%$$

$$SF = \frac{(\text{number of colonies formed after damage treatment})}{(\text{number of cells seeded})} \times 100\%$$

#### 13. Cell cycle analysis

Cells were harvested and washed with PBS. Pellets were fixed in ice-cold 70% ethanol and incubated at 4°C for at least 1hour. Washed cells with PBS for 2 times were collected by centrifugation at 850 g. Cells were resuspended with PBS diluted propidium iodide (PI) before analysis of cell cycle by FACSVerse.

14. Sample preparation for flow cytometry

Cells were harvested with trypsin and washed with PBS. Pellets were incubated in PBS-T (0.2% Triton X-100) at 4°C for 10 minutes. Cells then washed with 2% of FBS and incubated at 4°C for 20 minutes with BD cytofix/cytoperm buffer (BD Bioscience). BD perm/wash buffer (BD Bioscience) was used for washing. Samples were incubated with RPA antibody diluted in BD perm/wash buffer at RT for 2 hours. After wash twice with BD perm/wash buffer, Alexa-488 Donkey-anti-rabbit antibody diluted in BD perm/wash buffer was added for 20 minutes at 4°C. Samples were washed with BD perm/wash buffer. PI (0.2 µg/ml) and RNaseA (10 µg/ml) were added before analysis of fluorescence by FACSVerse.

15. High Content Screening (HCS)

Cells were seeded in SCREENSTAR 96-well plates (Greiner) and treated with small molecules before staining with S9.6 antibody. HCS was performed with ImageXpress Micro XLS. 16 images per well were acquired for both channels at 20X magnification. Images were analyzed using MetaXpress.



## Results

### Screening small molecules regulating R-loop formation

ATAD5 forms a Replication Factor C (RFC) Like Complex (RLC) and unloads PCNA during DNA replication [19]. Since ATAD5 stabilizes in response to DNA replication stresses, a HEK293T cell line expressing luciferase fused ATAD5 (ATAD5-luc) was established to detect DNA replication stress [20]. Previously, our laboratory used ATAD5-luc HEK293T cells to identify small molecules enhancing DNA replication stresses and found ~300 compounds. Since accumulated R-loop could cause replication stress and many DNA repair proteins might contribute to regulating R-loop formation, I hypothesized that identified small molecules would regulate R-loop formation. To establish R-loop detection with treatment of small molecules, CPT, which is a well-known R-loop inducing compound by inhibiting topoisomerase I, was employed to optimize R-loop detection method. I set up imaging and flow-cytometry methods for R-loop detection. R-loop detection by imaging was established with S9.6 antibody with confocal microscope (Figure 2A, B). Increased level of R-loop upon CPT treatment was established with imaging method (Figure 2A). Many R-loops were formed in the nucleolin which is consistent to the previous report (Figure2B, [21]). After checking CPT-induced R-loop formation with confocal microscope, the level of R-loop with CPT treatment was check using MetaXpress. The intensity of S9.6 increased with CPT treatment in concentration dependent manner and it peaked at 10  $\mu$ M of CPT treatment (Figure 2C, D). HB-GFP can indirectly detect R-loops in vivo [22] since HB-GFP binds to R-loop without resolving R-loop structure. I successfully detected R-loop with HB-GFP after CPT treatment (Figure 3A). In flow cytometry analysis, HB-GFP was used to indirectly mark the R-loop. The level of R-loop peaked at 15 minutes of 5  $\mu$ M CPT treatment (Figure 3B).

### Baicalein reduces CPT-induced R-loop formation

Baicalein is a type of flavonoid and it is an analogue of baicalin [23] (Figure 4a). Recently, my colleague also found that mismatch repair proteins, MSH2-MSH3 function at the early step of homologous recombination (Oh et al., unpublished results). Since MSH2 and baicalein interacts each other, I hypothesized that baicalein could enhance cellular toxicity caused by different DSB inducing DNA damaging agents. I tested CPT, etoposide or ionizing radiation, which induce DNA DSBs by inhibiting topoisomerase I, II, or energy transfer, respectively [24, 25]. Baicalein treatment together with CPT showed synergistic cellular death, which was not observed by etoposide or IR treatment (Figure 4b). CPT is a well-known DNA damage reagent by inhibiting topoisomerase I and also known to increase R-loop formation [26]. Therefore, I checked whether R-loop formation enhanced by CPT treatment is affected by co-treatment of baicalein. The level of R-loop was decreased upon co-treatment of baicalein and CPT compared to CPT treatment alone (Figure 4c, d). Consistent with the

R-loop formation, the level of RPA was also decreased when CPT was treated together with baicalein compared to CPT treatment alone. R-loops are mainly generated when transcription and replication collided each other. Thus, higher R-loop formation could result in S-phase delay [27]. However, co-treatment of baicalein and CPT did not show any significant change of S-phase population compared to CPT treatment alone (Figure 5a). Further FACS experiment checking delay of S-phase should be done with G1 phase arrest.

### **Baicalein didn't strongly bind to DNA:RNA hybrid**

Baicalein preferentially binds to mismatched dsDNA than to unmodified DNA [9]. Mismatched base pairs in dsDNA can form bubble structure [28], and R-loops also mimics bubble DNA structure [29]. Before checking whether baicalein can bind to R-loop structure, I checked whether baicalein preferentially bind to DNA:RNA hybrid. However, I could not detect preferential interaction of baicalein with DNA:RNA hybrid compared to double stranded DNA (Figure 6a, b). I will investigate whether baicalein preferentially interact with R-loop structure compared to normal DNA structure.

### **R-loop is resolved by baicalein in XPF dependent manner**

It was recently reported that baicalein induced  $\gamma$ -H2AX in mismatch repair deficient cells in XPF dependent manner [9]. Therefore, I hypothesized that XPF could function to reduce CPT-induced R-loop formation upon baicalein treatment. To test the hypothesis, I monitored the R-loop formation in XPF deficient cells in various treatment conditions with CPT and baicalein. The R-loop was increased with baicalein treatment with CPT in XPF deficient cells, whereas baicalein treatment with CPT significantly decreased the formation of R-loop formation in XPF complemented cells (Figure 7a, b). Surprisingly, the nuclease activity-dead mutant of XPF (D687A) rescued the level of R-loop formation upon both baicalein and XPF treatment similar to wild type complemented cells (Figure 7a, b). Therefore, I concluded that baicalein regulates the CPT-induced R-loop formation in XPF dependent manner but nuclease activity of XPF is dispensable (Figure 7c).

### **XPF is important to recruit 53BP1 to the chromatin by baicalein**

NHEJ and HR are predominant repair pathways for DSBs and determinants of each pathway are accumulation of 53BP1 at DSB sites and recruitment of BRCA1, respectively [12, 13]. Since improperly resolved R-loops can lead to DSBs [2, 3], the increased level of  $\gamma$ -H2AX and 53BP1 were monitored. The level of  $\gamma$ -H2AX and 53BP1 on chromatin enhanced by CPT was reduced upon baicalein treatment (Figure 8a). Consistently, the intensity of both  $\gamma$ -H2AX and 53BP1 nuclear foci was decreased upon baicalein and CPT treatments compared to CPT treatment alone (Figure 8b, c). However, the level of  $\gamma$ -H2AX and 53BP1 was further increased by baicalein and CPT co-treatment in in XPF deficient cells (Figure 8a). Consistently, immunoblotting of  $\gamma$ -H2AX and 53BP1 showed

similar results (Figure 8b, c). Collectively, 53BP1 recruitment to the chromatin and the enhanced level of  $\gamma$ -H2AX by CPT treatment are reduced by baicalein treatment in XPF dependent manner.

#### **Catalytic activity of XPF is dispensable for 53BP1 recruitment to the chromatin by baicalein**

Nuclease activity of XPF is important to function in DNA damage repair [30]. To validate if nuclease activity of XPF is important to resolve R-loop with baicalein, I used cells expressing XPF catalytic dead-mutant: D687A in patient derived XPF-deficient cells. XPF catalytic-dead mutant rescued the level of R-loop formation upon both baicalein and CPT treatment similar to wild type XPF (Figure 7a, b). Therefore, I checked whether XPF catalytic-dead mutant could also rescue the level of  $\gamma$ -H2AX and 53BP1 upon baicalein and CPT co-treatment in an XPF dependent manner by Western blotting and immunostaining. 53BP1 recruitment to chromatin was decreased upon baicalein treatment alone or with CPT treatment in XPF nuclease dead mutant cells. Surprisingly, similar to XPF WT, XPF mutant didn't have significant difference in the level of  $\gamma$ -H2AX upon treatment of CPT alone or CPT and baicalein. The level of 53BP1 also rescued the level of protein on chromatin by Western blot analysis (Figure 9a). The level of 53BP1 also rescued in XPF catalytic mutant cell lines in immunostaining data (Figure 9b, c). In conclusion, nuclease activity of XPF is not important for 53BP1 recruitment to chromatin upon baicalein treatment. Therefore, I will analyze if XPF is able to recruit other DNA repair proteins such as SLX4 for the resolution of R loop caused by CPT.

## Further study

Baicalein synergistically induces cell death with CPT (Figure 4A). Since I only checked the level of R-loop formation by baicalein and CPT, I will analyze the R-loop formation by baicalein with other DNA damaging reagents.

NER is divided into two sub pathways: GG-NER and TC-NER depending on the transcriptional activity of the chromosome [31]. Two nucleases XPF and XPG both work in GG-NER and TC-NER [11]. To validate the effect is specific on XPF or NER including both TC-NER and global NER, I will compare the R-loop formation in other NER protein, XPG deficient cells.

XPF can either work with XPA in NER or with SLX4 in the interstrand cross linking (ICL) repair. To further distinguish the effect is limited to NER or ICL, I will compare the R-loop formation in XPA deficient cells and SLX4 deficient cells.

To elucidate molecular mechanism how baicalein reduces CPT-induced R-loop formation and 53BP1 recruitment to chromatin in the XPF dependent manner, I need further following studies. First, I have to check the interaction between R-loop and XPF, R-loop and 53BP1 and XPF and 53BP1. I am going to investigate those interactions with Proximity Ligation assay (PLA), which can see the interaction between proteins. Also, there is no report that 53BP1 is linked to R-loop formation. R-loop formation in 53BP1 deficient condition will be investigated.

Baicalein binds to mismatched DNA preferentially. dsDNA having mismatched base pairs got bubble structure [9]. It seemed that baicalein could have binding affinity to bubble structures in R-loop structure. I am planning to conduct an experiment using SPR system to verify the interaction between baicalein and R-loop structure.

## Discussion & Conclusion

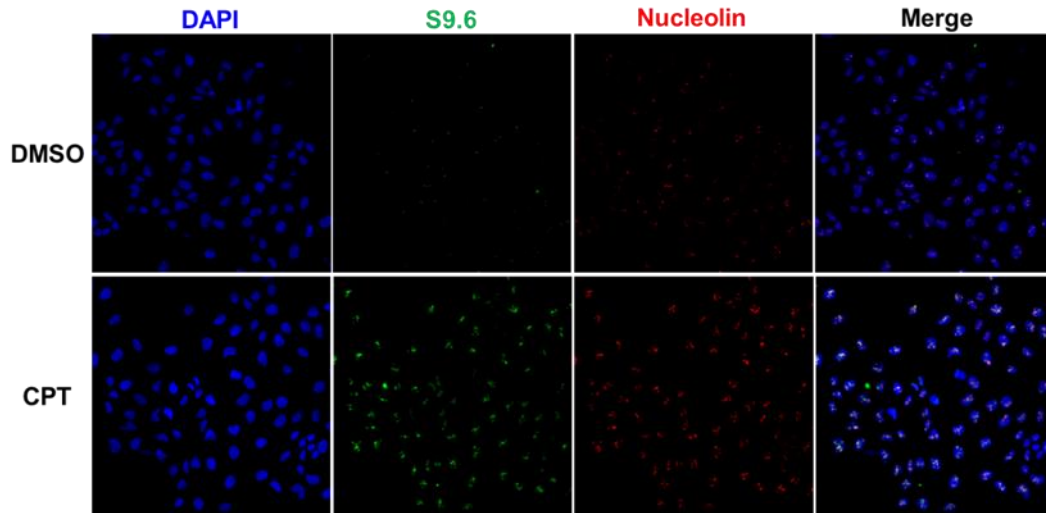
Regulation of R-loop formation should be tightly regulated as their integrity is involved in DNA damage, transcription and a lot of cellular function. Emerging studies of R-loop are focused on novel protein regulating R-loop formation. Small molecules inducing DNA damage can enhance abnormal R-loop formation. Since, dysregulation of R-loop formation in damage condition could increase genomic instability and potentially tumorigenesis, comprehensive understanding of R-loop formation and small molecules affecting R-loop formation would be beneficial to cancer patients. In this study, I found that baicalein can regulate R-loop formation without affecting the level of  $\gamma$ -H2AX (Figure 4c, d, Figure 8a, b, c). Baicalein regulates R-loop formation in XPF dependent manner. However, the catalytic activity of XPF appears not to be important for R-loop regulation (Figure 8A, B). Further functional study of XPF in R-loop with baicalein would reveal unknown function of XPF. There are several SLX4 mutations found in patients defective in XPF interaction [32]. It would be interesting to investigate whether R-loop regulation by XPF requires SLX4 interaction. Those mechanistic studies would verify the importance of XPF in R-loop formation.

53BP1 recruitment to chromatin was decreased by baicalein and it also seemed to depend on XPF (Figure 8a, b, c). The relationship between 53BP1 and XPF is not revealed and even whether 53BP1 has role in R-loop formation or not is not discovered so far. Study validating the direct mechanism between 53BP1 and XPF would discover novel mechanism of R-loop. Checking the relationship between 53BP1 and R-loop would contribute to reveal the role of novel DNA damage repair proteins in R-loop formation. It could unveil the potential role of NHEJ pathway in R-loop regulation.

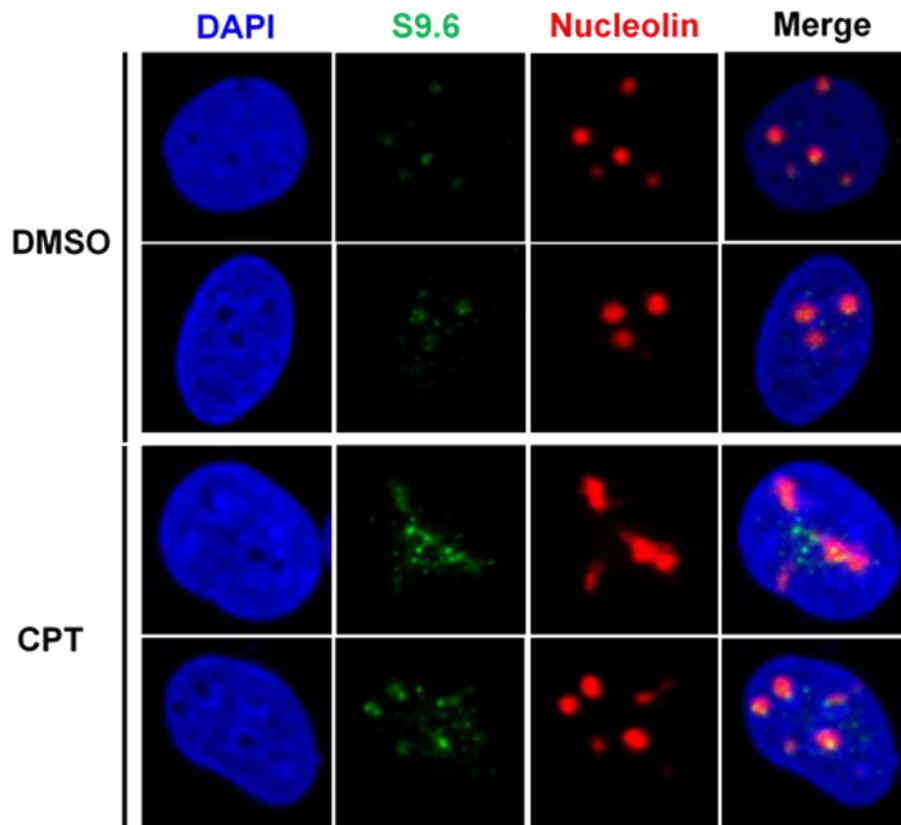
DNA damaging agent, CPT is commonly prescribed to cancer patients [25]. Patients with CPT-resistant cancers are prescribed CPT with other DNA toxins or irradiation therapy (CPT-resistance and additional therapy). Small molecules identified in following studies will benefit to CPT-resistant cancer patients. Furthermore, small molecules regulating R-loop formation can be the key for tumor patients having resistant to typical chemical treatment.

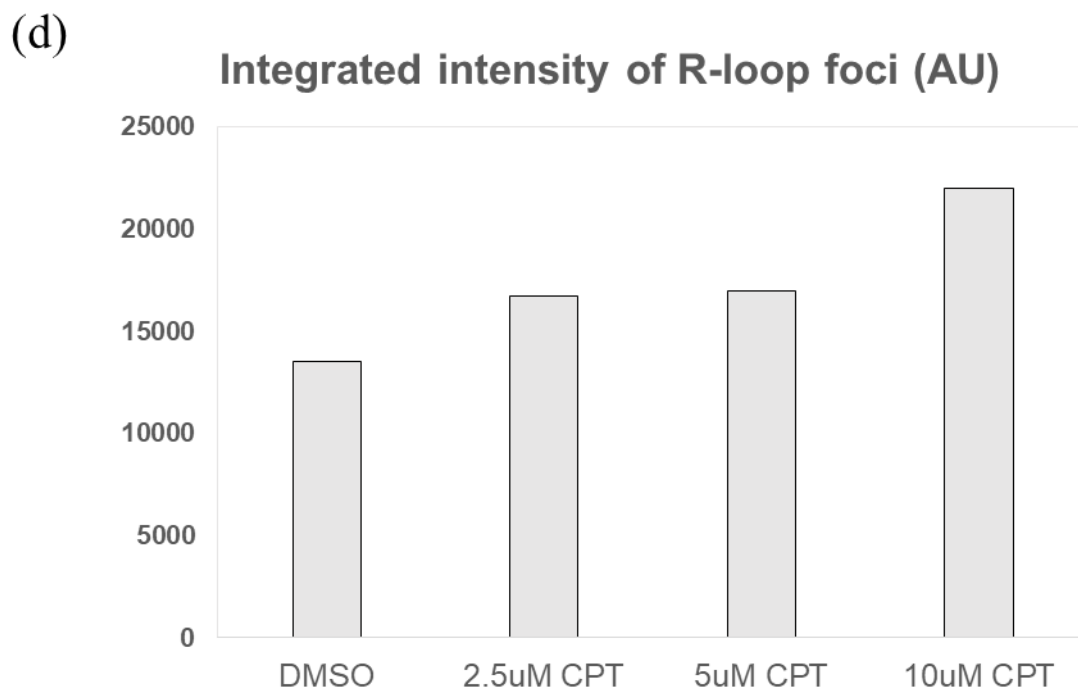
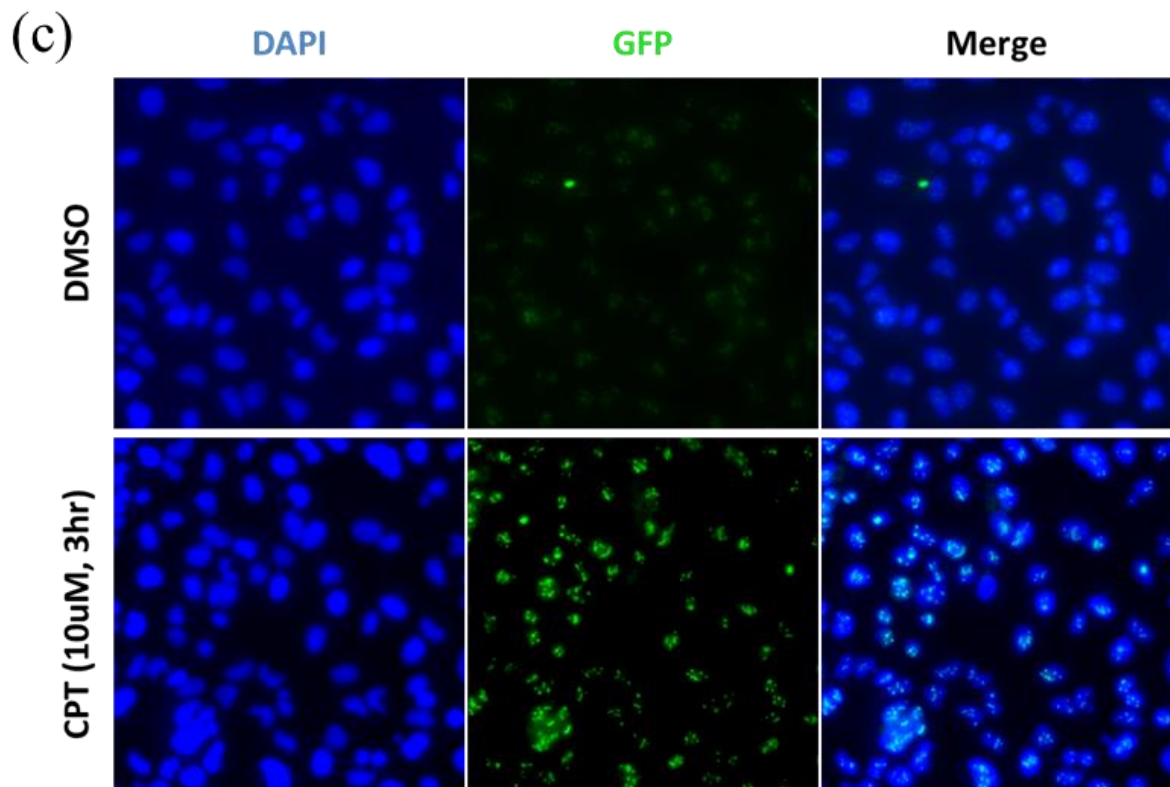
Most of the R-loop studies are focused on R-loops formed in cellular stress such as replication-transcription collision and DNA damage repair [1, 3]. Since R-loop formation is important for cellular processes [1-3], R-loops formed in non-DNA damage conditions should be studied. For most of the small molecules regulating R-loop formation induce DNA damage, further studies distinguish R-loop regulation effect and DNA damaging effect are needed to distinguish the cellular effects.

(a)



(b)

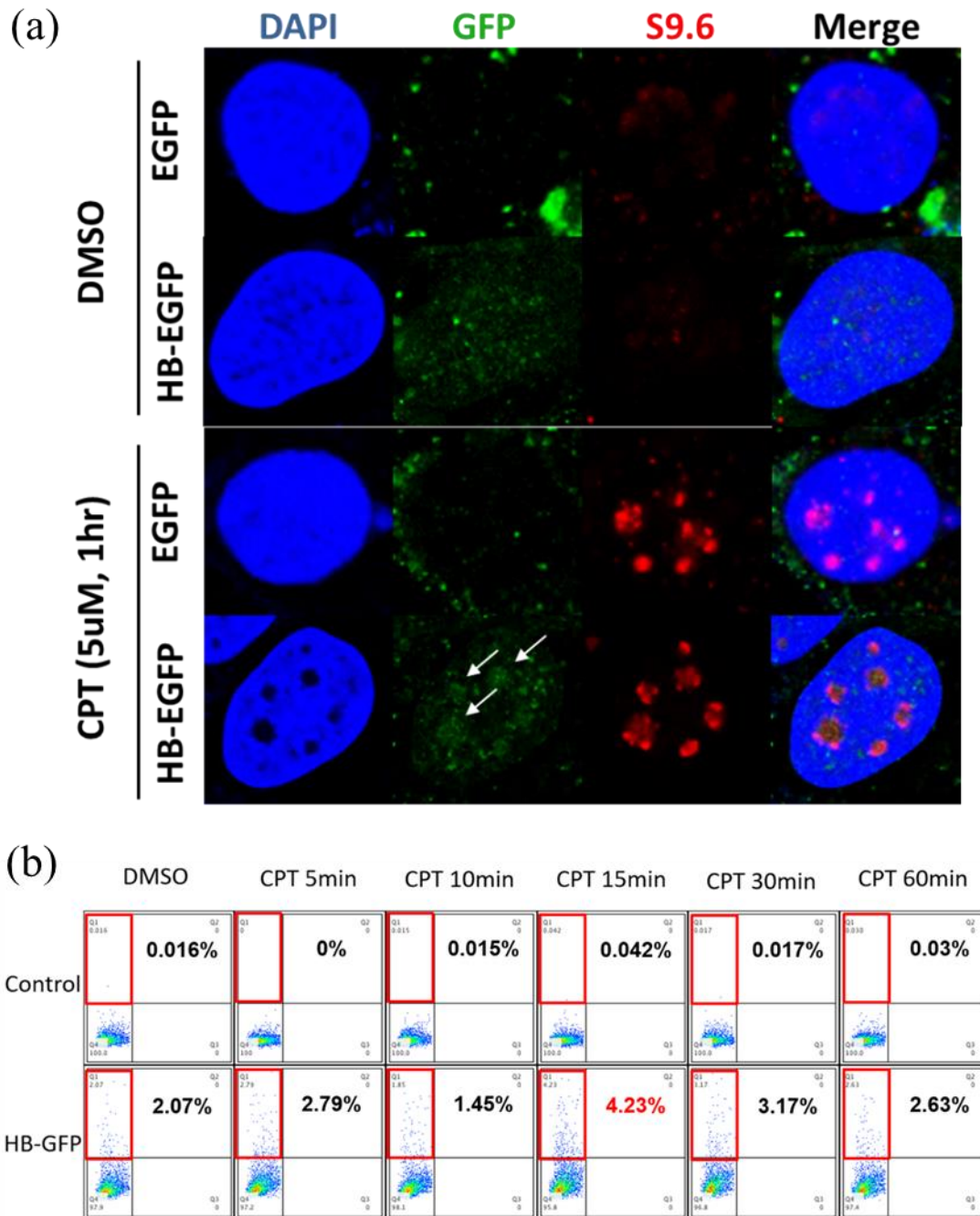




**Figure 2. Setting up HCS method using CPT as a positive control.**

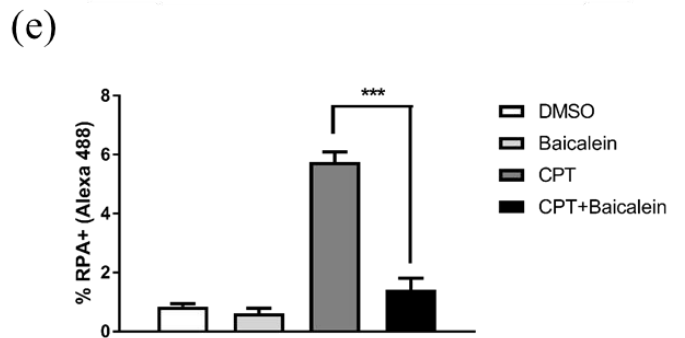
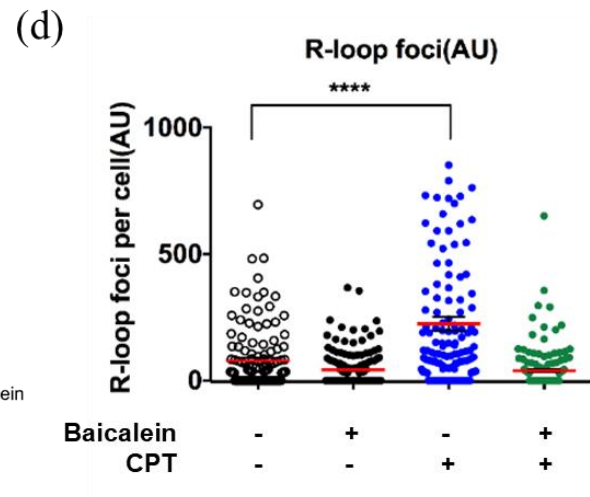
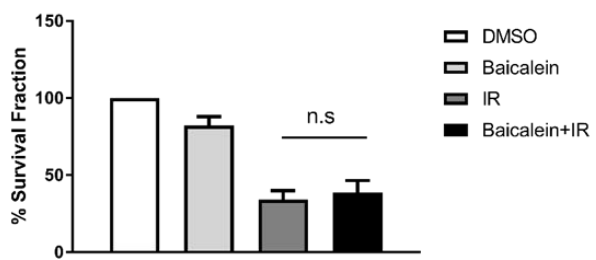
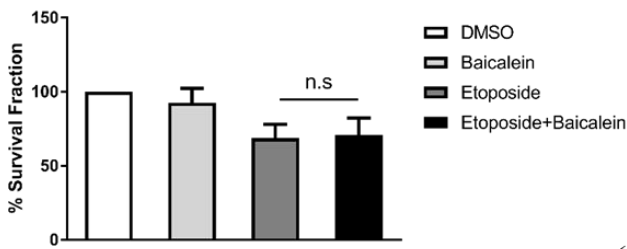
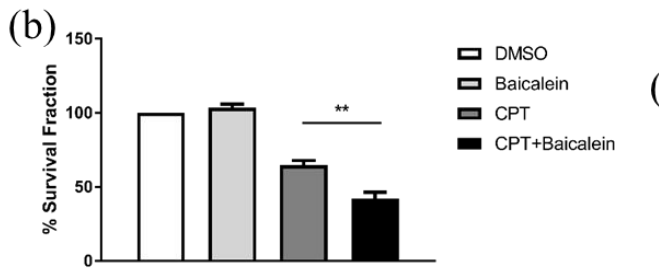
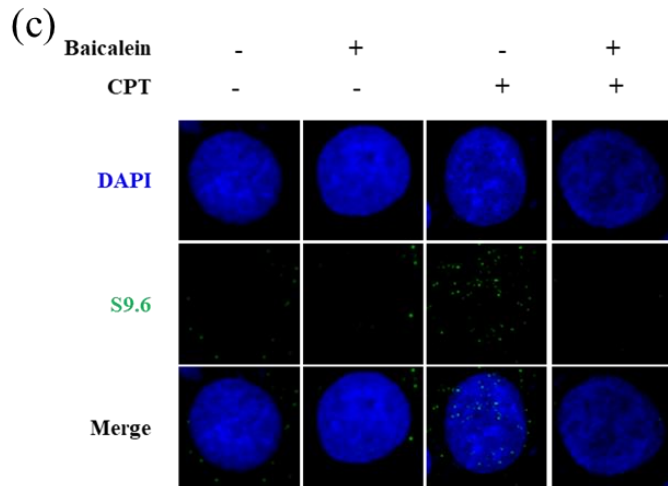
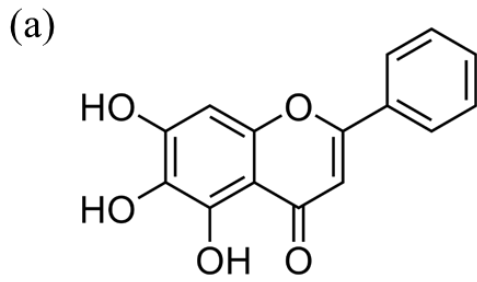
(a) 20X confocal image stained with S9.6 antibody. (b) 40X confocal image stained with S9.6 antibody. (c) Representative image obtained by MetaXpress. (d) Quantified HCS data with treatment of CPT.





**Figure 3. HB-GFP can be used as complementary method for R-loop screening.**

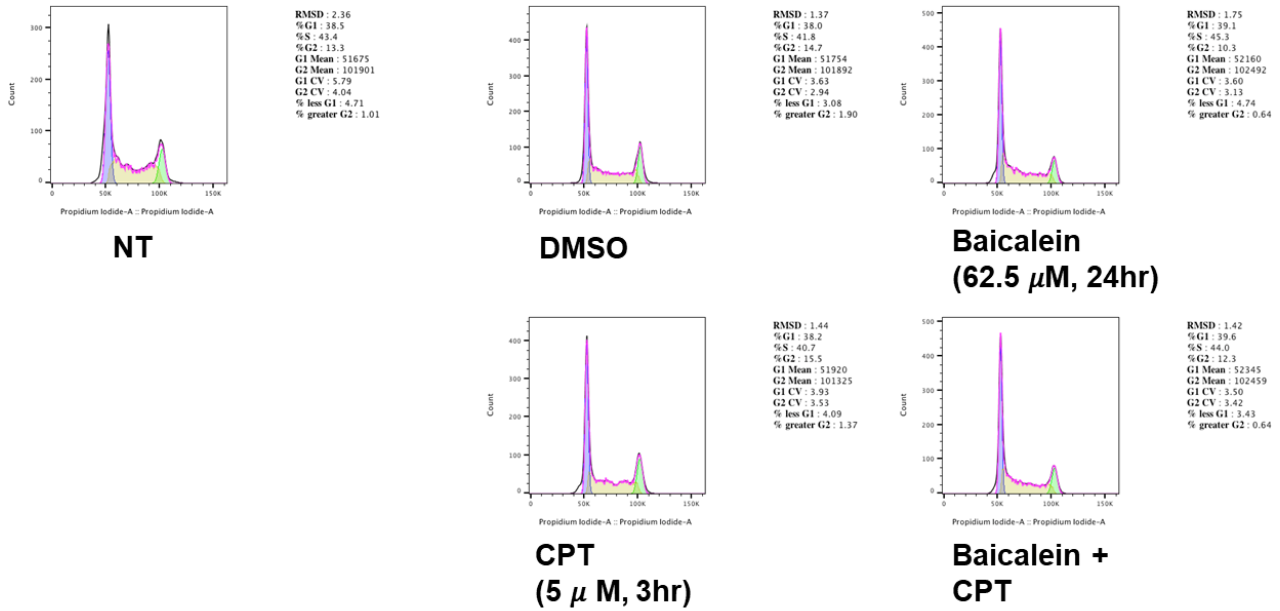
(a) HB-GFP and nucleolin were visualized upon CPT treatment. (b) FACS data upon CPT treatment in time-dependent manner.



**Figure 4. Baicalein reduced the CPT-induced R-loop formation.**

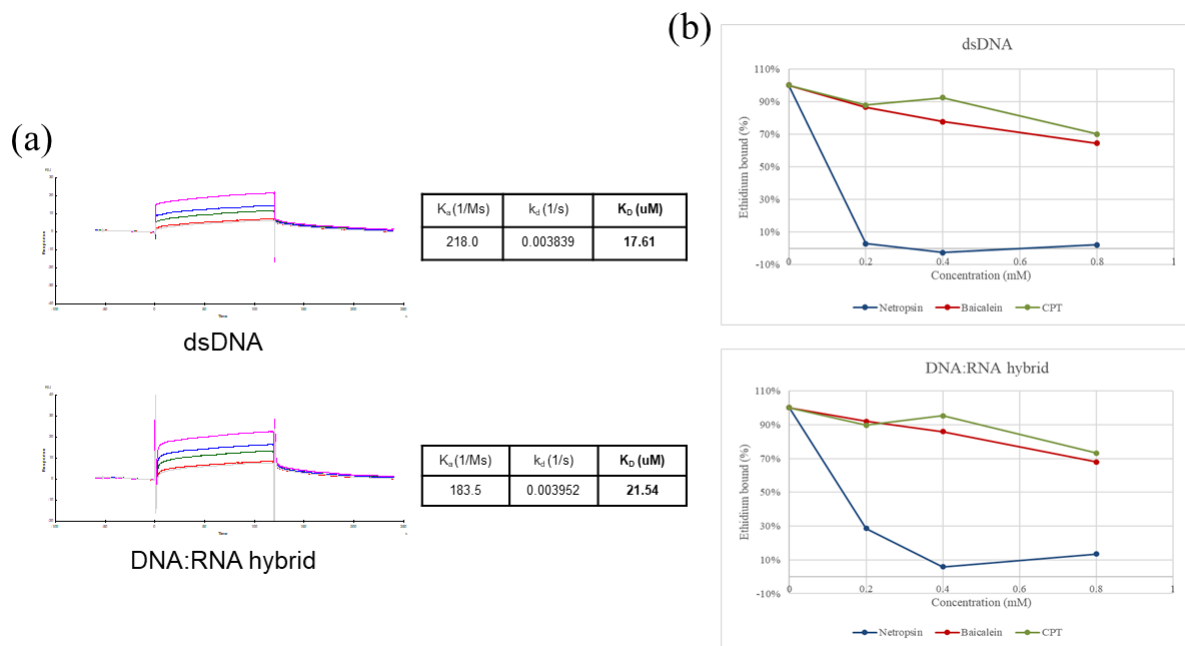
(a) Structure of baicalein (b) Colony forming assay to check the viability upon damage inducing reagents treatment with baicalein treatment. Co-treatment of CPT and baicalein further reduced the survival rate of U2OS. (\*\* :  $P < 0.005$ ) (c) Representative image of R-loop formation upon either baicalein or CPT treatment. R-loop was detected with S9.6 antibody. (d) Quantitative graph of (c). The intensity of total foci was analyzed with One-way ANOVA. (n=3, \*\*\*\* :  $P < 0.0001$ ) (e) The level of chromatin bound RPA was significantly reduced when baicalein was treated in CPT-treated U2OS cells. (\*\*\*) :  $P < 0.0005$ )

(a)



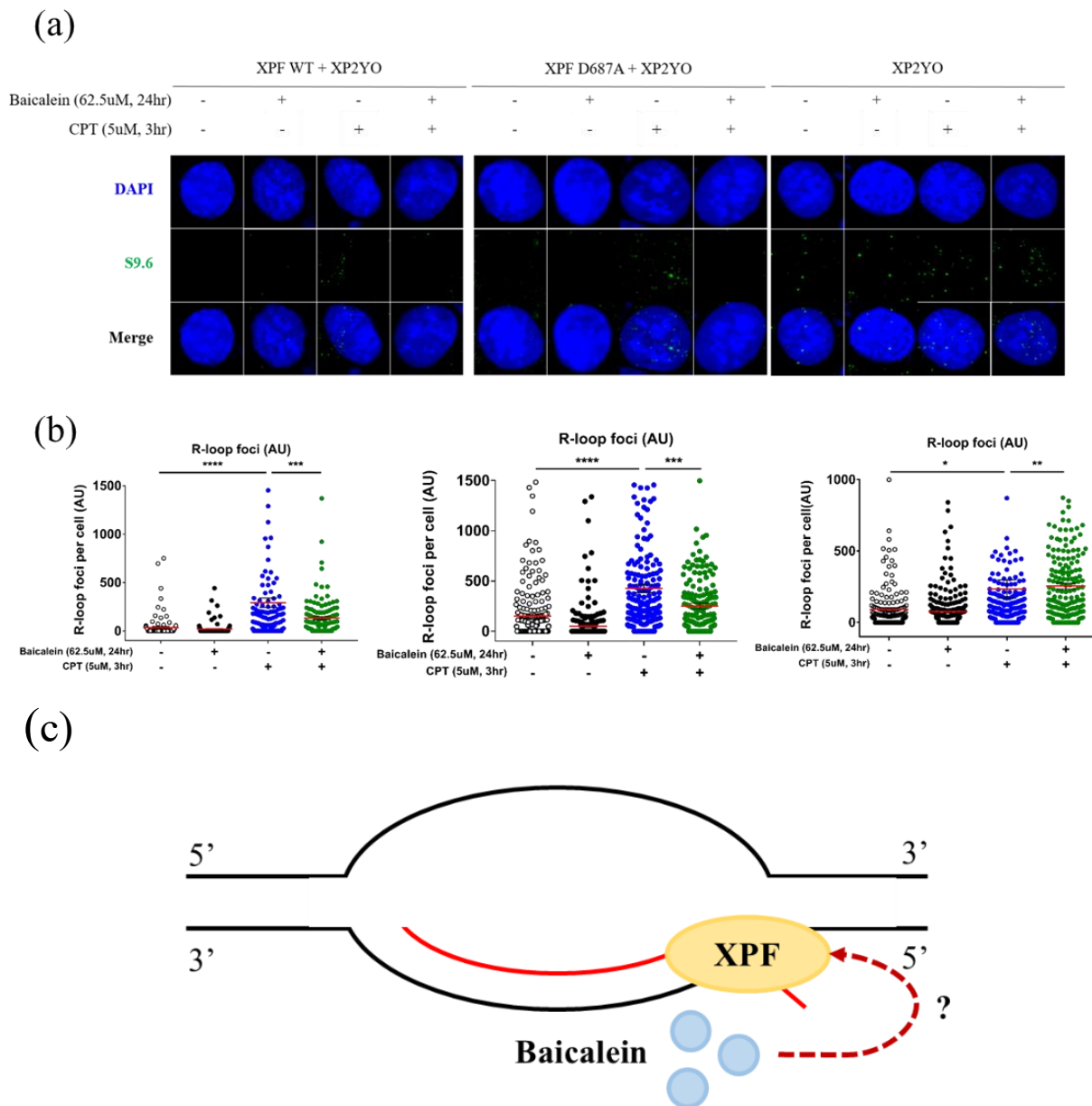
**Figure 5. Baicalein didn't affect cell cycle.**

(a) Cell cycles upon treatment of either baicalein or CPT are indicated. PI was used to quantify the amount of DNA contents.



**Figure 6. Baicalein doesn't preferentially bind to DNA:RNA hybrid.**

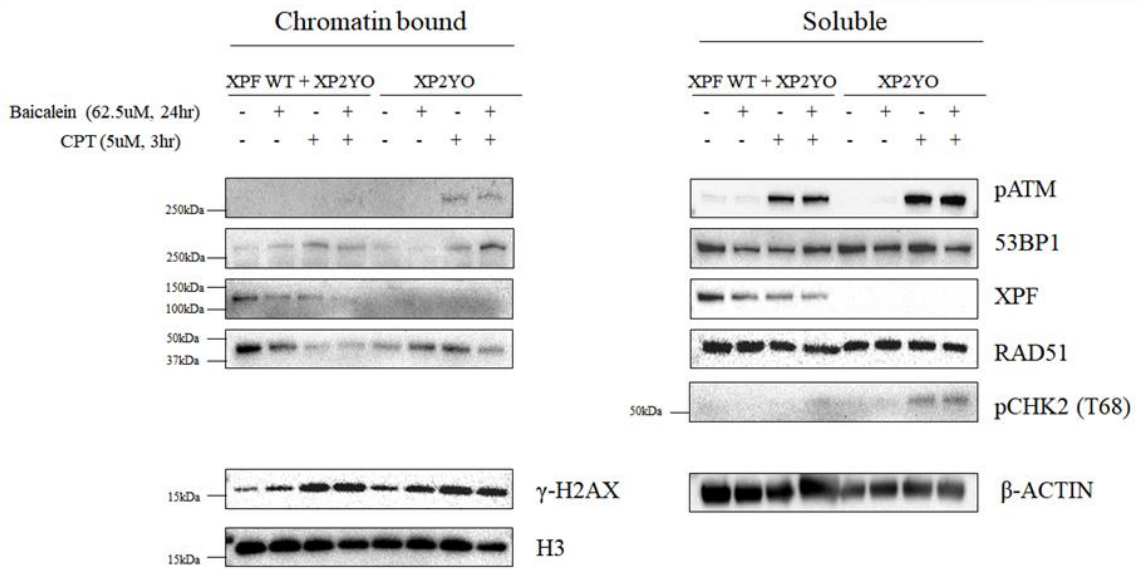
- (a) SPR system was used to check the interaction between baicalein and dsDNA or DNA:RNA hybrid.  
 (b) Interaction between baicalein and dsDNA or DNA:RNA hybrid were measured by the EtBr displacement assay.



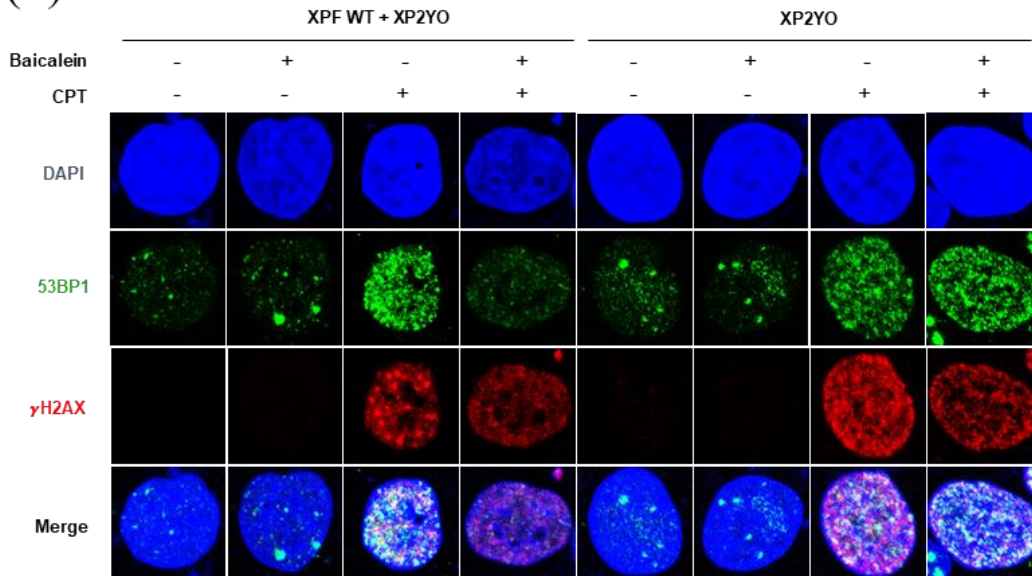
**Figure 7. Baicalein reduced the CPT-induced R-loop formation in an XPF dependent manner.**

(a) Representative image of R-loop formation upon either baicalein or CPT was detected with S9.6 antibody. (b) Quantitative data of (a). The intensity of total foci was analyzed with one-way ANOVA. (n=3, \* : P<0.05, \*\* : P<0.005, \*\*\* : P<0.0005, \*\*\*\* : P<0.0001) (c) Model of baicalein regulating R-loop formation through XPF.

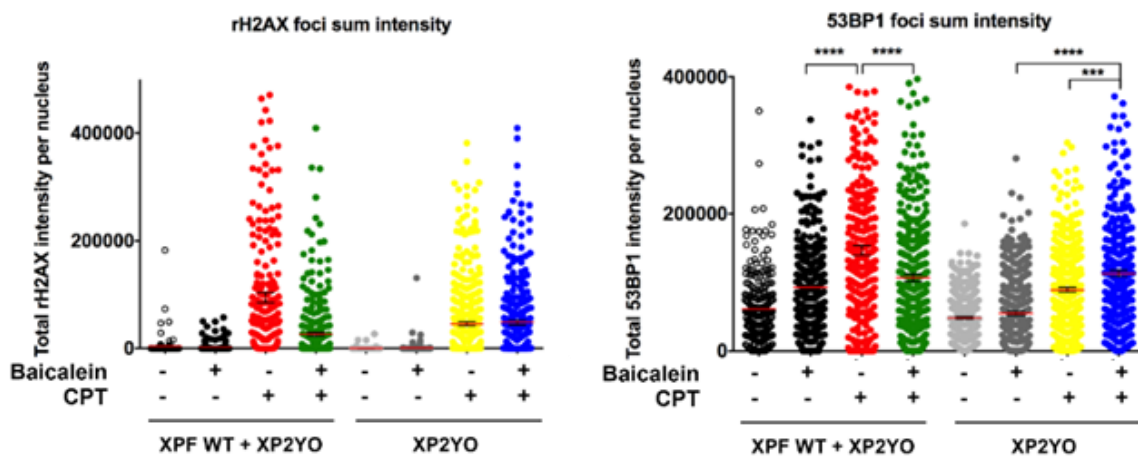
(a)



(b)



(c)

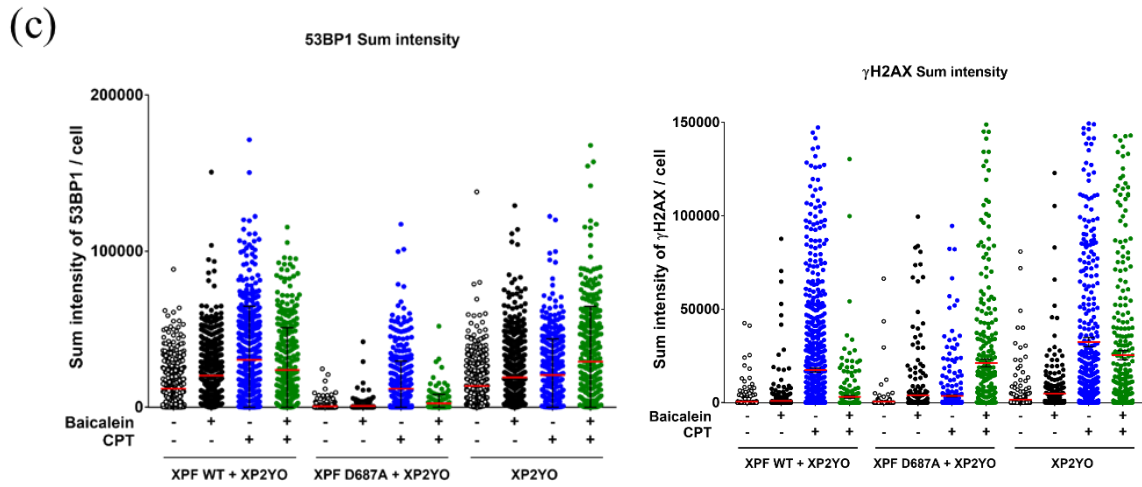


**Figure 8. Baicalein reduces the 53BP1 recruitment in an XPF dependent manner.**

(a) Chromatin bound 53BP1 are shown upon baicalein or CPT treatment. (b) Representative image of R-loop formation upon either baicalein or CPT was measured with S9.6 antibody. (c) Quantitative data of (b). The intensity of total foci was analyzed with One-way ANOVA. (n=3, \*\*\* : P<0.0005, \*\*\*\* : P<0.0001)







**Figure 9. Catalytic activity of XPF is dispensable for recruiting 53BP1 by baicalein.**

(a) Representative image of R-loop formation upon either baicalein or CPT was measured with S9.6 antibody. (b) Quantitative data of (a). (c) Quantitative data of (b). The intensity of total foci was analyzed with One-way ANOVA, \*\*\*\* :  $P < 0.0001$ .)

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