

Synergistic effect of inhibiting CHK2 and DNA replication on cancer cell growth

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eLife Assessment

This study presents **important** findings on the activity of two compounds, BKC and IBC, isolated from *Psoralea corylifolia*, which act synergistically to inhibit cancer cell proliferation. Using a spectrum of methods, the authors characterized the mechanisms of action of both drugs, providing **convincing** evidence that BKC targets DNA polymerases and IBC selectively inhibits CHK2. The study opens the possibility of improving the effectiveness of the combination of BKC and other damaging agents with IBC in cancer treatment.

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Abstract

Cancer cells display high levels of oncogene-induced replication stress (RS) and rely on DNA damage checkpoint for viability. This feature is exploited by cancer therapies to either increase RS to unbearable levels or to inhibit checkpoint kinases involved in the DNA damage response (DDR). Thus far, treatments that combine these two strategies have shown promise but also have severe adverse effects. To identify novel, better-tolerated anticancer combinations, we screened a collection of plant extracts and found two natural compounds from the plant, *Psoralea corylifolia*, that synergistically inhibit cancer cell proliferation. Bakuchiol inhibited DNA replication and activated the checkpoint kinase CHK1 by targeting DNA polymerases. Isobavachalcone interfered with DNA double-strand break (DSB) repair by inhibiting the checkpoint kinase CHK2 and DNA end resection. The combination of bakuchiol

and isobavachalcone synergistically inhibited cancer cell proliferation *in vitro*. Importantly, it also prevented tumor development in xenografted mice. The synergistic effect of inhibiting DNA replication and CHK2 signaling identifies a vulnerability of cancer cells that might be exploited by using clinically approved inhibitors in novel combination therapies.

Introduction

Genome integrity is particularly at-risk during S phase of the cell cycle, when thousands of replication forks travel at high speed along the chromosomes to duplicate the DNA. Replication initiates at specific sites called origins, which are sequentially activated throughout the length of the S phase (Fragkos *et al*, 2015 [↗](#)). Faithful DNA replication depends on the coordinated action of the many enzymes that make up the replisome (Attali *et al*, 2021 [↗](#)). During this process, the parental DNA strands are separated by the CMG helicase, consisting of CDC45, the MCM2-7 hexamer and the GINS complex. DNA is synthesized on the leading strand by DNA polymerase (Pol) ϵ and on the lagging strand by Pol α -primase and Pol δ (Lujan *et al*, 2016 [↗](#)).

Replication forks stall when they encounter obstacles such as DNA lesions, highly transcribed genes or tightly bound protein complexes, causing what is commonly referred to as replication stress (RS) (Lin & Pasero, 2021 [↗](#); Macheret & Halazonetis, 2015 [↗](#); Zeman & Cimprich, 2014 [↗](#)). RS is associated with an excess of single-stranded DNA (ssDNA), resulting from the uncoupling of DNA polymerase and helicase activities (Pasero & Vindigni, 2017 [↗](#)). Stalled or collapsed forks can also give rise to DNA double-strand breaks (DSBs). A signal transduction pathway called the intra-S checkpoint detects these stalled forks and associated DSBs (Zeman & Cimprich, 2014 [↗](#)). Upon fork arrest, ssDNA coated by the ssDNA-binding heterotrimer RPA recruits the Ser/Thr protein kinase ATR (Zou & Elledge, 2003 [↗](#)). Activation of ATR by TopBP1 initiates a signaling cascade involving phosphorylation and activation of the intra-S checkpoint kinases CHK1 and WEE1, which coordinate a variety of repair mechanisms to prevent fork collapse, resume DNA synthesis and delay entry into mitosis (Pasero & Vindigni, 2017 [↗](#); Saldivar *et al*, 2017 [↗](#); Stracker *et al*, 2008 [↗](#)). The presence of DSBs is also signaled by two other protein kinases, ATM and DNA-PK, which activate the checkpoint kinase CHK2 and result in cell cycle arrest or cell death (Blackford & Jackson, 2017 [↗](#)). ATR, ATM and DNA-PK phosphorylate the histone variant H2AX on S139 (γ -H2AX), which is a reliable biomarker of RS and DSBs (Bonner *et al*, 2008 [↗](#)).

During the S and G₂ phases of the cell cycle, DSBs are preferentially repaired by homologous recombination (HR), which uses the sister chromatid as repair template (Moynahan & Jasin, 2010 [↗](#)). In G₁ phase or in HR-deficient cells, DSBs are repaired by more error-prone pathways such as non-homologous end joining (NHEJ) and single-strand annealing (SSA) (Chang *et al*, 2017 [↗](#)). HR-mediated DSB repair is initiated with the resection of DNA ends to generate 3'-protruding extremities that are coated by the RAD51 recombinase to form a RAD51 filament involved in homology search (Cejka & Symington, 2021 [↗](#); Chakraborty *et al*, 2023 [↗](#)). RAD51 loading depends on BRCA1 and BRCA2, which also play a role at stalled replication forks to prevent the hyper-resection of nascent DNA (Tye *et al*, 2020 [↗](#)).

In precancerous lesions, deregulated oncogenic pathways perturb the proper execution of the DNA replication program, leading to increased fork collapse and chromosome breaks (Macheret & Halazonetis, 2015 [↗](#)). This oncogene-induced RS, on the one hand, promotes cancer development by increasing genomic instability and promoting the loss of p53 (Halazonetis *et al*, 2008 [↗](#)), on the other hand, it is a burden for cancer cells, which must deal with chronic replication defects and may become dependent on checkpoint function for their survival. This burden can be exploited for cancer treatment by the use of genotoxic drugs that further increase RS and/or by inhibiting the ATR pathway (Lecona & Fernandez-Capetillo, 2018 [↗](#); Ubhi & Brown, 2019 [↗](#); Zhu *et al*, 2020 [↗](#)). Cancer cells eventually adapt to oncogene-induced RS, however, by overexpressing downstream components of the ATR pathway, such as Claspin, Timeless and CHK1, which correlates with poor

prognosis in breast, lung and colon carcinomas (Bianco *et al*, 2019 [↗](#)). Thus, genotoxic agents used to increase RS in cancer cells are generally effective as first-line treatments to reduce tumor mass, but patients often relapse as the cancer cells become resistant to treatment.

Since cancer cells depend on an effective RS response for their survival, considerable effort has been made to develop small-molecule inhibitors of the intra-S checkpoint kinases ATR, CHK1 and WEE1 (Dobbelstein & Sørensen, 2015 [↗](#); Lecona & Fernandez-Capetillo, 2018 [↗](#); Ubhi & Brown, 2019 [↗](#)). In principle, inhibiting these kinases should selectively kill cancer cells that have elevated levels of RS while sparing healthy cells. Several inhibitors have now entered clinical trials, with mixed results (Bradbury *et al*, 2020 [↗](#); Gorecki *et al*, 2021 [↗](#)). ATR inhibitors are usually well tolerated when used in monotherapy, but they have limited efficacy because low ATR activity can be compensated by ATM. ATR inhibitors are more effective when combined with subtherapeutic doses of chemotherapeutic agents (e.g. gemcitabine), but at the expense of serious adverse effects, such as myelosuppression (Dobbelstein & Sørensen, 2015 [↗](#); Gorecki *et al*, 2021 [↗](#); Nazareth *et al*, 2019 [↗](#)). CHK1 inhibitors are toxic, especially when used in combinatorial therapies (Neizer-Ashun & Bhattacharya, 2021 [↗](#)). WEE1 inhibitors are better tolerated when used in monotherapy and combination regimens, but clinical trials have shown only modest benefits so far (Gorecki *et al*, 2021 [↗](#)). Thus, although some of these inhibitors are promising, they are still far from ready to be used in the clinic. Further efforts are still needed to identify new small molecule inhibitors, or combinations of inhibitors, that target the RS response in cancer cells but are not excessively toxic to normal cells.

To identify novel small molecules that target the RS response in cancer cells, we screened crude extracts of plants used in traditional Chinese herbal medicine for their ability to kill cancer cells selectively by inducing RS. An extract from the plant *Psoralea corylifolia* showed the most promising anticancer activity. We show that bakuchiol (BKC) and isobavachalcone (IBC), two compounds isolated from this extract, act synergistically to prevent the proliferation of cancer cells, by inhibiting DNA synthesis and by impeding the resection of DNA ends at DSBs, respectively. Together, these compounds reduce tumor growth and improve survival in a xenograft mouse model and IBC alone potentiates the anticancer effect of chemotherapeutic agents in diffuse large B-cell lymphoma cells. The synergistic effect of an inhibitor of DNA synthesis and an inhibitor of DNA end resection identifies a novel vulnerability of cancer cells that might be exploited by using clinically approved inhibitors of these mechanisms in novel combination therapies.

Results

IBC and BKC synergistically inhibit proliferation of cancer cell lines

To identify novel combinations of small molecule inhibitors that target DNA replication in cancer cells, we screened a selection of crude extracts of Chinese herbal medicines for their ability to differentially impede cell growth and induce γ -H2AX foci in MCF-7 human breast cancer cells relative to non-cancerous BJ hTERT-immortalized human fibroblasts. In an extract prepared from *Psoralea corylifolia*, we identified two compounds, isobavachalcone (IBC; MW: 324.4; (Kwete & Sandjo, 2012 [↗](#))) and bakuchiol (BKC; MW: 256.4; (Nizam *et al*, 2023 [↗](#)); **Fig. 1A** [↗](#)), which inhibited the proliferation of MCF-7 cells and A549 human lung cancer cells more than they did the proliferation of BJ fibroblasts and non-transformed epithelial cells MCF10A and RPE-1 cells (**Fig. 1B** [↗](#), **S1A** [↗](#) and **S1B** [↗](#)). BKC is a bioactive meroterpene that possesses a variety of pharmacological activities (Xin *et al*, 2019 [↗](#)). It was shown to inhibit the proliferation of many cancer cell lines (Li *et al*, 2016 [↗](#)) presumably through the inhibition of DNA replication (Sun *et al*, 1998 [↗](#)). IBC is a natural chalcone that also exhibits potential anticancer activities (Kwete *et al*, 2015 [↗](#); Ren *et al*, 2024 [↗](#); Wu *et al*, 2022 [↗](#)). However, the combined anticancer effect of BKC and IBC has never been addressed and their mechanisms of action and molecular targets have remained unknown.

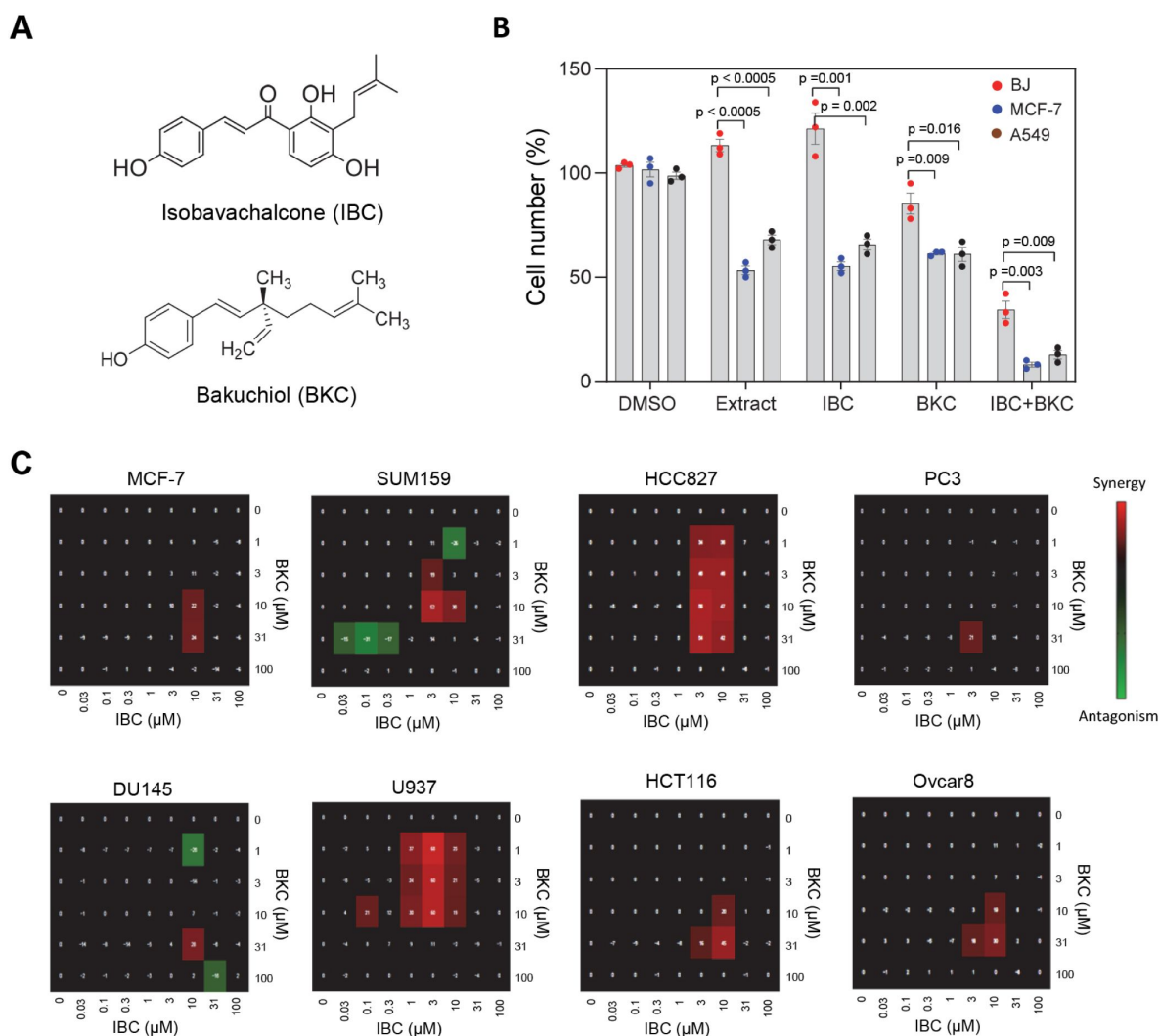


Fig. 1.

IBC and BKC synergistically inhibit proliferation of cancer cell lines.

A. Chemical structures of isobavachalcone (IBC) and bakuchiol (BKC). **B.** BJ, MCF-7 and A549 cells were treated with DMSO, 25 $\mu\text{g/ml}$ crude extract (PR7), 15 μM IBC, 40 μM BKC or the combination 15 μM IBC and 40 μM BKC for 72 hours. These concentrations were used throughout the study. Cell number was quantified by using the WST-1 assay. Data are means $\pm$ SD of three independent experiments. The p-values were calculated by two-tailed unpaired t-test. **C.** Concentration matrix analyses of a panel of eight cancer cell lines treated with IBC and BKC at the indicated doses for 72 hours. Cell viability was measured by using the sulforhodamine B colorimetric assay. Antagonist combinations (green), synergistic combinations (red) and additive effects (black) were calculated. A representative analysis of three independent experiments is shown.

Here, we have used a panel of eight tumor cell lines including two breast (MCF-7 and SUM159), one lung (HCC827), two prostate (PC3 and DU145), one lymphoma (U937), one colon (HCT116) and one ovarian (Ovcar8) cancer cell lines to investigate the antitumoral effect of IBC and BKC. We first determined the IC_{50} of each compound in each cell line (**Fig. S1C**) and found that IBC was more potent than BKC in inhibiting the proliferation of all cell lines. We then evaluated the effect of combined use of IBC and BKC on the viability of the eight cell lines by using a concentration matrix approach and a quantitative colorimetric cytotoxicity assay (**Fig. S1D**) from which we calculated the synergistic and antagonistic effects of the two compounds (**Fig. 1C**), as previously described (Tosi *et al.*, 2018). In all cell lines tested, we observed a synergistic effect of IBC and BKC over a narrow range of concentrations (3–10 μ M IBC and 10–30 μ M BKC) and an additive effect of the two drugs over a wider concentration range. This ratio of 3:10 μ M IBC: BKC that has a synergistic effect *in vitro* corresponds to the ratio of concentrations of the two compounds in the fruits of *P. corylifolia*. We conclude from this data that IBC and BKC inhibit cell growth in a synergistic manner and that this effect is more pronounced on cancer cells than on non-cancer cells.

IBC and BKC induce replication stress

To study the potential of IBC and BKC to induce RS, we assayed formation of γ -H2AX foci in MCF-7 and BJ cells treated for 24 hours with DMSO or with the two compounds, either alone or in combination. Cells were labeled with the thymidine analogue 5-ethynyl-2-deoxyuridine (EdU) to identify cells in S phase and γ -H2AX foci were detected by immunofluorescence microscopy; γ -H2AX levels in S phase cells was quantified as mean fluorescence intensity. IBC increased γ -H2AX signal in MCF-7 but not in BJ cells, whereas BKC increased it in both cell types (**Fig. 2A** and **2B**). Moreover, the combination of IBC and BKC further increased γ -H2AX fluorescence in MCF-7 but not in BJ cells (**Fig. 2A** and **2B**), which is consistent with their effect on cancer cell growth. BKC alone or in combination with IBC also induced phosphorylation of CHK1 on S345 in MCF-7 cells, whereas IBC alone did not (**Fig. 2C** and **S2B**). These data suggest that BKC, but not IBC, can directly induce replication stress.

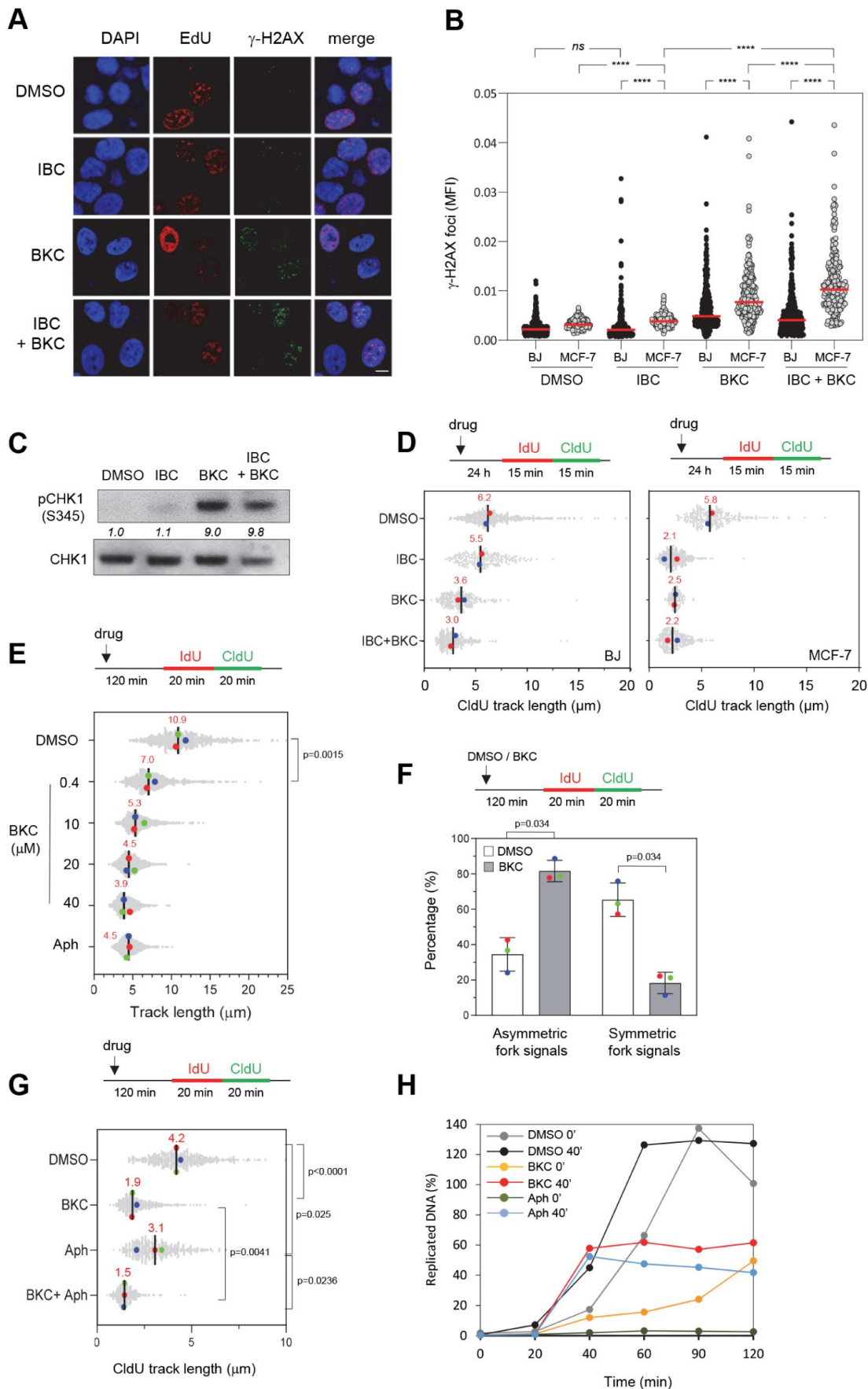


Fig. 2.

IBC and BKC induce replication stress and impede fork progression.

A. MCF-7 and BJ cells were treated with either 15 μ M IBC, 40 μ M BKC or both (IBC + BKC) for 24 hours, then 10 μ M EdU was added for 10 minutes and γ -H2AX foci in EdU-positive cells were detected by using Click chemistry and immunofluorescence microscopy. Representative immunofluorescence images of MCF-7 cells are shown. Bar: 5 μ m. **B.** Mean fluorescence intensity (MFI) of γ -H2AX foci was quantified by CellProfiler. One of three independent experiments is shown (n=3). ****, $p < 0.0001$; ns, not significant, Mann-Whitney rank sum test. **C.** MCF-7 cells were treated with IBC/BKC for 24 hours, as in panel A, and CHK1 phosphorylated on S345 (pCHK1) was detected by western blotting. The ratio of pCHK1 to total CHK1, relative to the DMSO control, is indicated. A representative example of two independent experiments is shown. **D.** BJ and MCF-7 cells were treated with IBC/BKC for 24 hours, as in panel A, then IdU and CldU were added sequentially each for 15 minutes. Replication fork progression was determined by measuring CldU track lengths in DNA fiber spreads. The median length of CldU tracks is indicated in red. At least 150 fibers were measured for each condition. Median of two independent experiments is indicated in red. **E.** MCF-7 cells were treated with indicated concentrations of BKC or 1 μ M Aphidicolin for 2 hours then IdU and CldU were added sequentially each for 20 minutes. Replication fork progression was determined as in panel D. The length of IdU and CldU was measured. Median of three independent experiments is indicated in red. The p-values were determined by two-tailed unpaired t-test (n=3). **F.** MCF-7 cells were treated with DMSO or 20 μ M BKC for 2 hours then IdU and CldU were added sequentially each for 20 minutes. Replication fork progression was determined as indicated in panel D. The ratio of the CldU signal of two sister forks was calculated. At least 80 sister forks were measured in each biological replicate. The ratio of two sister forks between 0.8-1.2 was considered as symmetric forks. Mean + SEM of three independent experiments are shown. The p-values were determined by two-tailed unpaired t-test. **G.** MCF-7 cells were treated with 40 μ M BKC, aphidicolin (Aph, 10 μ M) or both (BKC + Aph) for 2 hours, prior to DNA fiber spreading assay. The p-values were determined by two-tailed unpaired t-test (n=3). **H.** *Xenopus* egg extracts were incubated with demembranated sperm nuclei and treated immediately (0 min) or after 40 minutes (40 min) with DMSO, BKC (100 μ M) or aphidicolin (Aph, 60 μ M). Samples were collected at the indicated time points after addition of the sperm nuclei. The percentage of replicated DNA was calculated as described in the Materials and Methods.

To address this possibility, we measured the effect of IBC and BKC on replication fork progression in MCF-7 and BJ cells by using a DNA fiber spreading assay. Briefly, the cells were exposed to one or both drugs for 24 hours and then labelled sequentially with the thymidine analogues 5-iodo-2'-deoxyuridine (IdU) and 5-chloro-2'-deoxyuridine (CldU) each for 15 minutes and the length of replicated tracks was measured along individual DNA fibers (**Fig. 2D**). Analysis of CldU track length showed that BKC reduced fork speed by a factor of two relative to untreated cells in both cell lines, suggesting that it directly inhibits DNA synthesis. In contrast, IBC inhibited fork speed more in MCF-7 cells than in BJ cells, indicating that it may affect DNA synthesis through a mechanism different from that of BKC. When used in combination, the inhibitory effect of BKC and IBC on fork speed was further increased, consistent with our finding that these drugs have a synergistic effect on cell growth.

To evaluate the impact of IBC and BKC on the cell cycle, we exposed MCF-7 and BJ cells to these drugs for 24 hours, labelled the cells in S phase with EdU for 30 minutes and analyzed the distribution of cells in the various phases of the cell cycle by flow cytometry (**Fig. S2A**). Treatment with BKC, but not IBC, resulted in a greater proportion of both cell types in S phase, which is consistent with our observation that BKC, but not IBC, induced CHK1 activation (**Fig. 2C** and **S2B**). Together, these data suggest that IBC and BKC induce replication stress through different mechanisms to prevent cancer cell proliferation.

BKC inhibits DNA replication

Our data suggest that BKC might be a potent inhibitor of replication fork progression *in vivo* (**Fig. 2D**). That acts directly on replicative DNA polymerases. Using DNA fiber assay, we found that BKC inhibited fork progression in a dose-dependent manner (**Fig. 2E**). Furthermore, BKC treatment significantly increased sister fork asymmetry compared to DMSO-treated control cells (**Fig. 2F**). We then compared its effect to that of aphidicolin, a well-characterized inhibitor of DNA polymerases α , δ and ϵ (Cheng & Kuchta, 1993). MCF-7 cells were treated with 40 μ M BKC, 10 μ M aphidicolin, or both for two hours and replication fork progression was measured by DNA fiber spreading. Remarkably, BKC inhibited fork progression more effectively than aphidicolin at these concentrations and the combined effect of both compounds was similar to the effect of BKC alone (**Fig. 2G**). BKC is a phenolic compound structurally related to resveratrol. Since resveratrol was shown to induce replication stress by inhibiting dNTP synthesis (Benslimane *et al*, 2020; Fontecave *et al*, 1998), we tested the possibility that BKC might also impede DNA replication by inhibiting dNTP synthesis. To this end, we tested its effect on a replication assay in *Xenopus* egg extracts, which contain high concentrations of dNTPs and do not depend on dNTP synthesis to sustain effective DNA replication. In this assay, demembranated sperm nuclei incubated in egg extracts decondense, assemble pre-replication complexes within 20 minutes and initiate synchronous DNA synthesis (Mechali & Harland, 1982). When added at the start of the assay (0 min), BKC (100 μ M) exhibited a strong inhibitory effect on DNA replication, although not as profound as the effect of aphidicolin (60 μ M). Moreover, when added after 40 minutes, long after initiation of replication, BKC was as effective as aphidicolin (**Fig. 2H**), suggesting that it inhibits elongation. Consistent with this possibility, BKC effectively inhibited replication of ssDNA, a process that relies entirely on priming and elongation of DNA chains by replicative DNA polymerases (**Fig. S2C**).

We also performed *in silico* molecular docking to study the potential interaction of BKC with the catalytic subunits of DNA polymerases δ and ϵ . This analysis showed that BKC can occupy the deoxycytidine sites of both enzymes (**Fig. 3A** and **3B**). Using the Cellular Thermal Sensitivity Shift Assay (CETSA) (Martinez Molina *et al*, 2013), we found that BKC interacts with the catalytic subunits of Pol δ and Pole in MCF-7 cells to stabilize the thermal sensitivity of both proteins (**Fig. 3C** and **3D**). Importantly, BKC specifically stabilize the catalytic subunit POLD1 of Pol δ , but not that of the accessory subunit POLD3 in *Xenopus* egg extracts (**Fig. S3A** and **S3B**). Similarly, BKC did not alter the thermal sensitivity of *Xenopus* PCNA (**Fig. S3C**). Together, these findings indicate that BKC strongly inhibits DNA replication *in vivo* and *in vitro*, most likely by directly inhibiting DNA polymerases.

IBC specifically inhibits CHK2

Previous studies found that IBC impedes cell proliferation by inhibiting AKT, a protein kinase involved in cell survival and in the transcriptional regulation of DNA replication-associated genes (Jing *et al*, 2010; Spangle *et al*, 2016). To determine whether AKT inhibition accounts for the effect of IBC on DNA replication in MCF-7 cells, we assayed the effect of IBC on the auto-phosphorylation of endogenous AKT at S473 and compared it to the effect of an allosteric AKT inhibitor, MK-2206. Whereas MK-2206 strongly inhibited AKT phosphorylation, IBC had no effect when used at the concentration that inhibited cell proliferation (30 μ M), nor did BKC or a combination of both compounds (**Fig. S4A**). Moreover, unlike MK-2206, IBC had little or no effect on expression of the cell cycle genes encoding E2F1, E2F2 and PCNA (**Fig. S4B**). Also, unlike IBC, MK-2206 had no effect on replication fork speed (**Fig. S4C**). We conclude that, at the concentrations used in this study, IBC inhibition of DNA replication cannot be explained by inhibition of AKT.

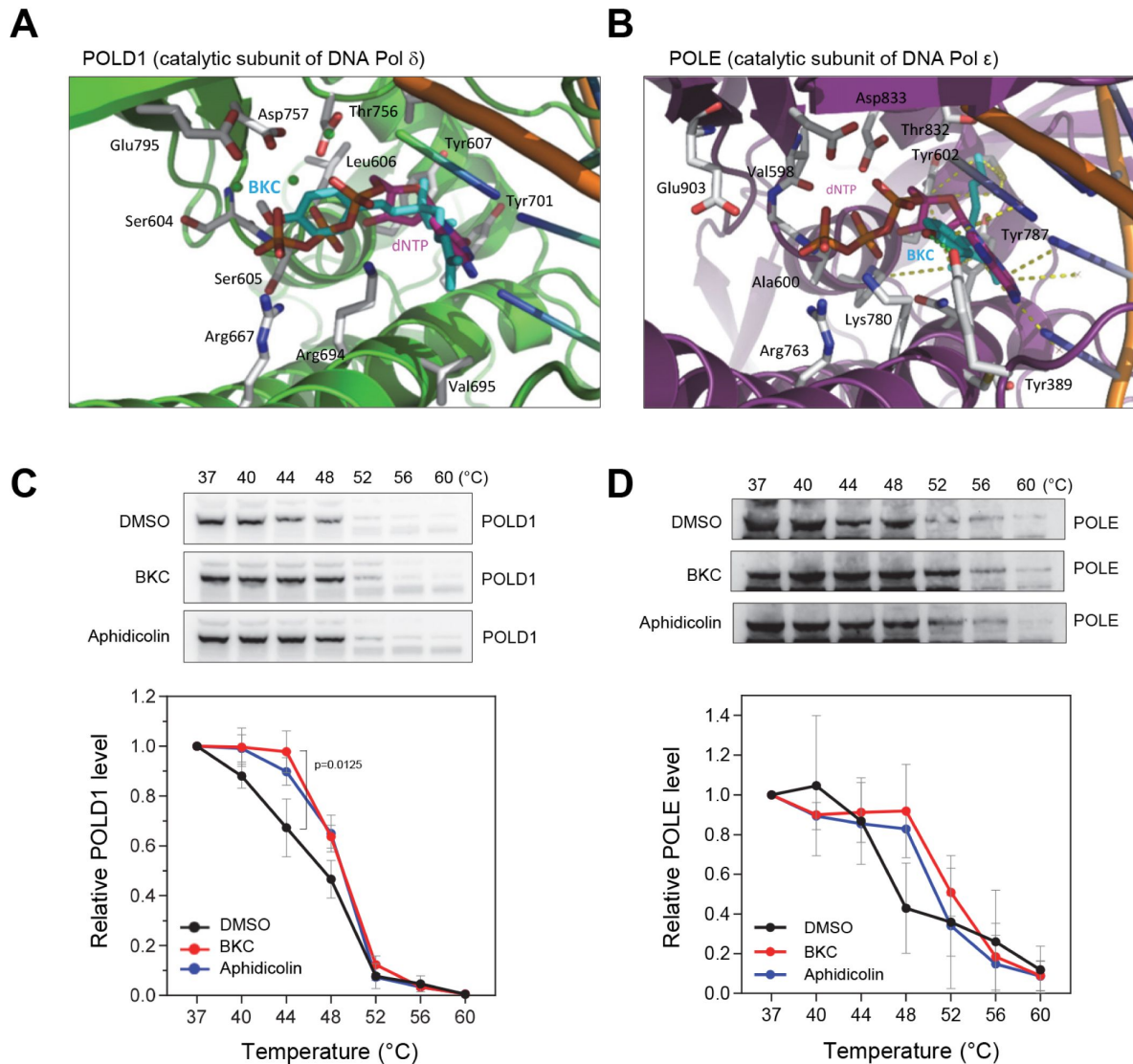


Fig. 3.

BKC directly binds to DNA polymerases.

A-B. *In silico* molecular docking of bakuchiol in the predicted active site structures of human DNA Pol δ and ϵ , respectively. **C-D.** MCF-7 cells were treated with DMSO, 40 μ M BKC or 10 μ M Aphidicolin for 2 hours prior to the cellular thermal sensitivity shift assay (CETSA) at indicated temperature, as described in the Materials and Methods. Levels of POLD1 (Pol δ ; panel C) and POLE (Pol ϵ ; panel D) catalytic subunits were detected by western blotting. Mean and SEM of three independent experiments are shown. The p-values were determined by two-tailed paired t-test.

To identify candidate target(s) of IBC that are responsible for its inhibitory effect on cancer cell proliferation, we assayed the effects of a broad range of IBC concentrations on the activities of 43 cell cycle-related kinases *in vitro* and determined the IC_{50} for each of them (Fig. 4A, S4D and S4E). The most sensitive kinase, CHK2, had an IC_{50} for IBC of 3.5 μ M (Fig. 4A). Aurora-A/B and JNK3 were also sensitive to IBC, but at approximately five-fold higher concentrations, IC_{50} of 11.2 μ M for Aurora-A/B and 16.4 μ M for JNK3. By contrast, CHK1 was not inhibited by IBC (Fig. 4A). Consistent with our findings above, the IC_{50} for AKT1/PKB α was 56.7 μ M (Fig. 4A, Fig. S4E and S4F).

To validate the inhibitory effect of IBC on CHK2 *in vivo*, we analyzed the auto-phosphorylation of CHK2 on S516 induced by camptothecin (CPT), a DNA topoisomerase I inhibitor that induces RS and DSBs (Pommier, 2006). In MCF-7 cells, IBC inhibited the auto-phosphorylation of CHK2 induced by CPT by approximately 50% (Fig. 4B and S4G). Consistently, IBC also inhibited the activation of the downstream target of CHK2, BRCA1. We showed that treatment with IBC reduced the phosphorylation of chromatin-bound BRCA1 at residue S988 induced by CPT, as efficiently as the commercial CHK2 inhibitor BML-277 (Fig. 4C and S4H). By contrast, IBC did not affect the autophosphorylation of CHK1 on S296 induced by hydroxyurea (HU; Fig. 4D and S4I). Using *in silico* molecular docking, we found that IBC binds to the active site of CHK2 by means of a hydrogen bond and 11 hydrophobic interactions (Fig. 4E), whereas its binding to the active site of CHK1 is prevented by a steric clash with a tyrosine residue (Tyr86) (Fig. 4F). To confirm the direct interaction between CHK2 and IBC, we performed the Cellular Thermal Shift Assay (CETSA) in MCF-7 cells. We showed that IBC altered the thermal stability of CHK2 as efficiently as the commercial CHK2 inhibitor, BML-277, without affecting the thermal stability of CHK1 (Fig. 4G and 4H). Interestingly, BML-277 seemed to have a slight effect in reducing the thermal stability of CHK1, although it was not statistically significant (Fig. 4G and 4H). Together, these data indicate that IBC inhibits CHK2 without affecting CHK1 activity.

IBC delays repair of DSBs induced by camptothecin

CHK2 promotes HR-mediated DSB repair (Parameswaran *et al*, 2015; Zhang *et al*, 2004). To investigate the effect of IBC on DSB repair, we induced chromosome breaks in MCF-7 cells by using camptothecin (CPT) and monitored the persistence of unrepaired DSBs in the following G_1 phase by immunofluorescence microscopy of 53BP1 foci and co-staining with an antibody against p27, a marker of G_1 cells. In cells treated with IBC, the CPT-induced 53BP1 foci persisted, whereas in cells not treated with IBC, the intensity of the camptothecin-induced 53BP1 immunofluorescence signal increased and returned to basal levels 24 hours later (Fig. 5A and 5B), indicating that IBC delays DSB repair. We also observed this persistence of DSBs in the presence of IBC by using pulsed-field gel electrophoresis (Fig. S5A), confirming that IBC impairs DSB repair, likely by inhibiting CHK2.

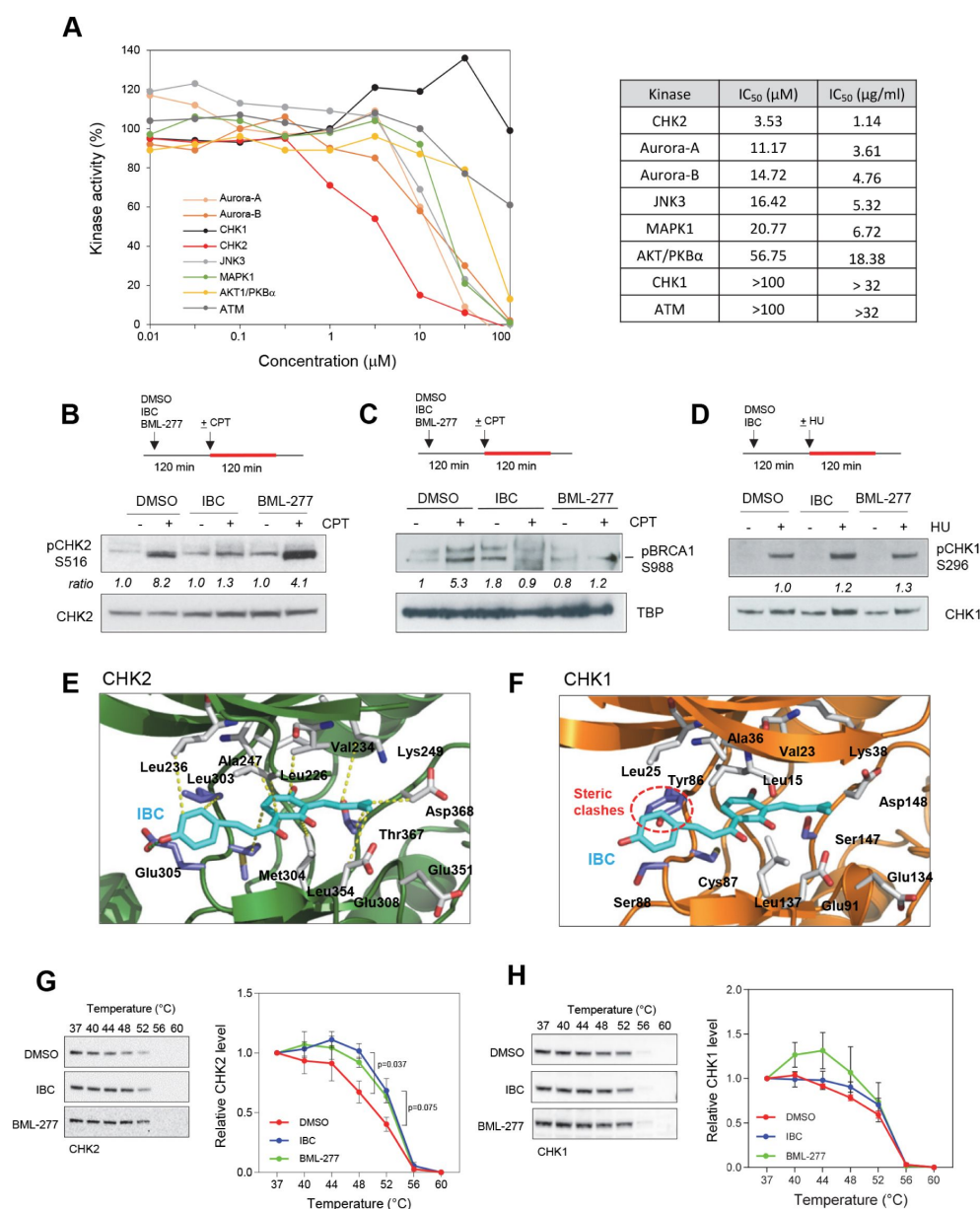


Fig. 4.

IBC inhibits the CHK2 kinase.

A. Selected protein kinases were incubated with the indicated range of IBC concentrations and kinase activity *in vitro* was determined by using a radiometric assay. The IC₅₀ of IBC for each kinase is indicated in the panel on the right. **B.** MCF-7 cells were pre-treated with DMSO, 15 μM IBC, or 20 μM BML-277 for 2 hours, then camptothecin (CPT, 1 μM) was added for 2 hours. Phosphorylation of CHK2 on S516 (pCHK2) was detected by western blotting. The ratio of pCHK2-S516 induction, relative to the DMSO+CPT control, is indicated. (n=3) **C.** MCF-7 cells were treated as indicated in panel B. Phosphorylation of chromatin-bound BRCA1 at residue S988 was detected by western blotting. The relative ratio of pBRCA1-S988 signal, after normalization to ponceau signal, is indicated. TBP was used as a marker of chromatin fraction **D.** MCF-7 cells were treated with 15 μM IBC for 2 hours, then 4 mM HU was added for 2 hours. CHK1 auto-phosphorylation on S296 (pCHK1) was detected by western blotting. **E-F.** *In silico* molecular docking of IBC in the active sites of CHK2 and CHK1, respectively. **G-H.** Cellular Thermal Shift Assay (CETSA) of IBC on the thermal stability of CHK2 and CHK1. MCF-7 cells were treated with 15 μM IBC or 20 μM BML-277 for 2 hours. Cells were proceeded to CETSA as described in the Materials and Methods. The amount of CHK2 and CHK1 present in the supernatant was detected by western blotting. The relative CHK2 and CHK1 signal was quantified. The p-values were determined by two-tailed paired t-test (n=3).

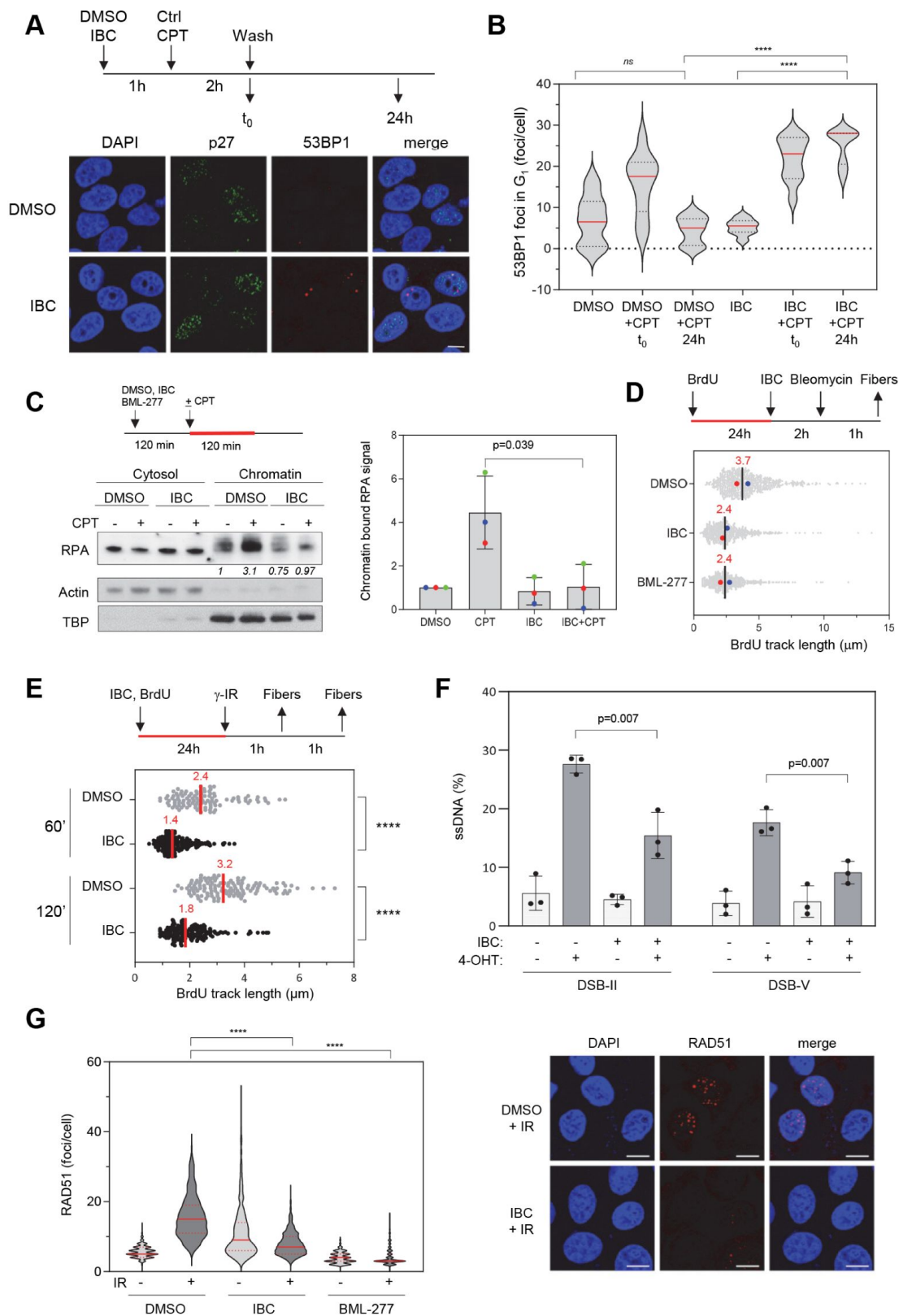


Fig. 5.

IBC impedes DNA end resection and RAD51 foci formation.

A. MCF-7 cells were treated with DMSO or 15 μ M IBC for 1 hour, then camptothecin (CPT, 1 μ M) was added for 2 hours. The cells were fixed immediately or washed and allowed to recover in medium containing DMSO or IBC for 24 hours before fixation. 53BP1 foci in p27-positive nuclei were detected by immunofluorescence microscopy. Representative images are shown. Bar: 5 μ m. **B.** 53BP1 foci number was quantified by using CellProfiler. Representative data from three independent experiments are shown. ****, $p < 0.0001$; ns, not significant, Mann-Whitney rank sum test. **C.** MCF-7 cells were treated with DMSO or 15 μ M IBC for 2 hours, then camptothecin (CPT, 1 μ M) was added for 2 hours, as indicated. Cells were fractionated into cytosol and nuclei (chromatin) and RPA in both fractions was detected by western blotting. Actin and TBP were used as markers of cytosol and chromatin fractions, respectively. The fold change of the chromatin-bound RPA signal relative to the DMSO control was quantified. The p-value was determined by unpaired t-test. (n=3) **D.** MCF-7 cells were incubated with 10 μ M BrdU for 24 hours to label genomic DNA, then DMSO, 15 μ M IBC or the CHK2 inhibitor BML-277 (20 μ M) were added for 2 hours followed by addition of 5 μ g/ml bleomycin for 1 hour. DNA fibers were spread on glass slides and BrdU was detected by immunofluorescence microscopy without DNA denaturation. The length of BrdU tracks was measured and the median for each condition is indicated in red. At least 250 fibers were measured for each condition. Median of two independent experiments is indicated in red (n=2). **E.** MCF-7 cells were incubated with 10 μ M BrdU for 24 hours to label genomic DNA in the presence of DMSO or IBC then exposed to ionizing radiations (8 Gy). Cells were collected 60 or 120 minutes after irradiation and BrdU tracks measured as in part B. ****, $p < 0.0001$, Mann-Whitney rank sum test. **F.** DivA cells were treated with either DMSO or IBC for 2 hours then DNA breaks were induced by treatment with 300 nM 4-hydroxytamoxifen (4-OHT) for 4 hours. Resection at two break sites, DSB-II and DSB-V, was determined as the percentage of ssDNA at these sites, calculated as indicated in the Material and Methods. Data are means $\pm$ SD (n=3). The p-values are indicated (two-tailed paired t-test). **G.** MCF-7 cells were treated with DMSO, IBC or BML-277 for 2 hours, then irradiated as described above. After 1 hour, RAD51 foci were detected by CSK-immunofluorescence microscopy and foci number was quantified using CellProfiler. Representative data (left) and immunofluorescence images (right) from two independent experiments are shown. Bar, 10 μ m. ****, $p < 0.0001$, Mann-Whitney rank sum test.

IBC prevents DNA end resection at DSBs

CHK2 phosphorylates BRCA1 on S988 to stimulate HR-mediated DSB repair (Parameswaran *et al.*, 2015; Zhang *et al.*, 2004). Since BRCA1 promotes DNA end resection at DSBs to initiate HR, we investigated whether IBC might impede the formation of single-strand DNA (ssDNA) at DNA ends. To do so, we induced DSBs by treating MCF-7 cells with camptothecin and assayed the formation of ssDNA by monitoring binding of the ssDNA-binding factor RPA to chromatin by western blotting and by immunofluorescence microscopy. These analyses revealed that IBC inhibited the formation of RPA-coated ssDNA upon CPT treatment (Fig. 5C and Fig. 55B).

To determine whether this effect was due to inhibition of DNA end resection, we used Single-Molecule Analysis of Resection Tracks (SMART) (Cruz-García *et al.*, 2014). In this assay, the length of BrdU-labeled ssDNA tracks exposed by resection is measured after induction of DSBs in MCF-7 cells with bleomycin. We found that the BrdU tracks were significantly shorter when CHK2 was inhibited either by IBC or by the CHK2 inhibitor BML-277 (Arienti *et al.*, 2005) (Fig. 5D). Using the same assay, we found that IBC also impaired DNA end resection induced by gamma irradiation (Fig. 5E). Furthermore, we confirmed that IBC prevents DSB end resection by using the DivA system, in which site-specific DSBs are generated by the restriction enzyme *AsiSI* to allow the quantification of ssDNA generated at break sites by quantitative PCR (Iacovoni *et al.*, 2010). IBC inhibited the formation of ssDNA at two break sites induced by *AsiSI* digestion (Fig. 55C and 5F), indicating that inhibition of CHK2 by IBC inhibits DSB end resection. Moreover, the extent of inhibition by IBC was similar to that caused by BML-277 (Fig. 55C).

To analyze the consequences of CHK2 inhibition by IBC on HR, we assayed formation of RAD51 foci following induction of DSBs by ionizing radiation (8 Gy) or by bleomycin treatment. CHK2 inhibition by either IBC or BML-277 completely prevented formation of RAD51 foci in response to ionizing radiation (**Fig. 5G** [↗](#)) and IBC completely prevented formation of RAD51 foci in response to bleomycin (**Fig. S5D** [↗](#)). Of note, the number of breaks induced by bleomycin was similar in cells treated with IBC or DMSO (**Fig. S5E** [↗](#)).

IBC and BKC synergistically inhibit tumor growth and extend survival in mice

To determine whether IBC and BKC might prevent cancer cell growth *in vivo*, we used a mouse xenograft model in which MCF-7 cells harboring an integrated firefly luciferase gene (MCF-7/Luc cells) were injected subcutaneously into the fat pads of NOD/SCID mice. Two days later, randomized mice were injected subsequently three times per week with two different concentrations of IBC, BKC or IBC+BKC, or with Taxol as a positive control, or with PBS as a negative control. Tumor size was measured at intervals of one week up to three weeks by measuring bioluminescence intensity and survival rate was monitored daily up to 70 days (**Fig. 6A** [↗](#)). After three weeks, IBC and BKC significantly inhibited tumor growth in a dose-dependent manner (**Fig. 6B** [↗](#) and **Fig. S6A** [↗](#)). IBC had a stronger effect on the growth of MCF-7/Luc cells than BKC had, but the combined use of both compounds had the greatest inhibitory effect on tumor growth (**Fig. 6B** [↗](#)). Moreover, the mice injected with the higher dose of IBC or of the IBC+BKC combination survived longer than those treated with Taxol (**Fig. 6C** [↗](#)).

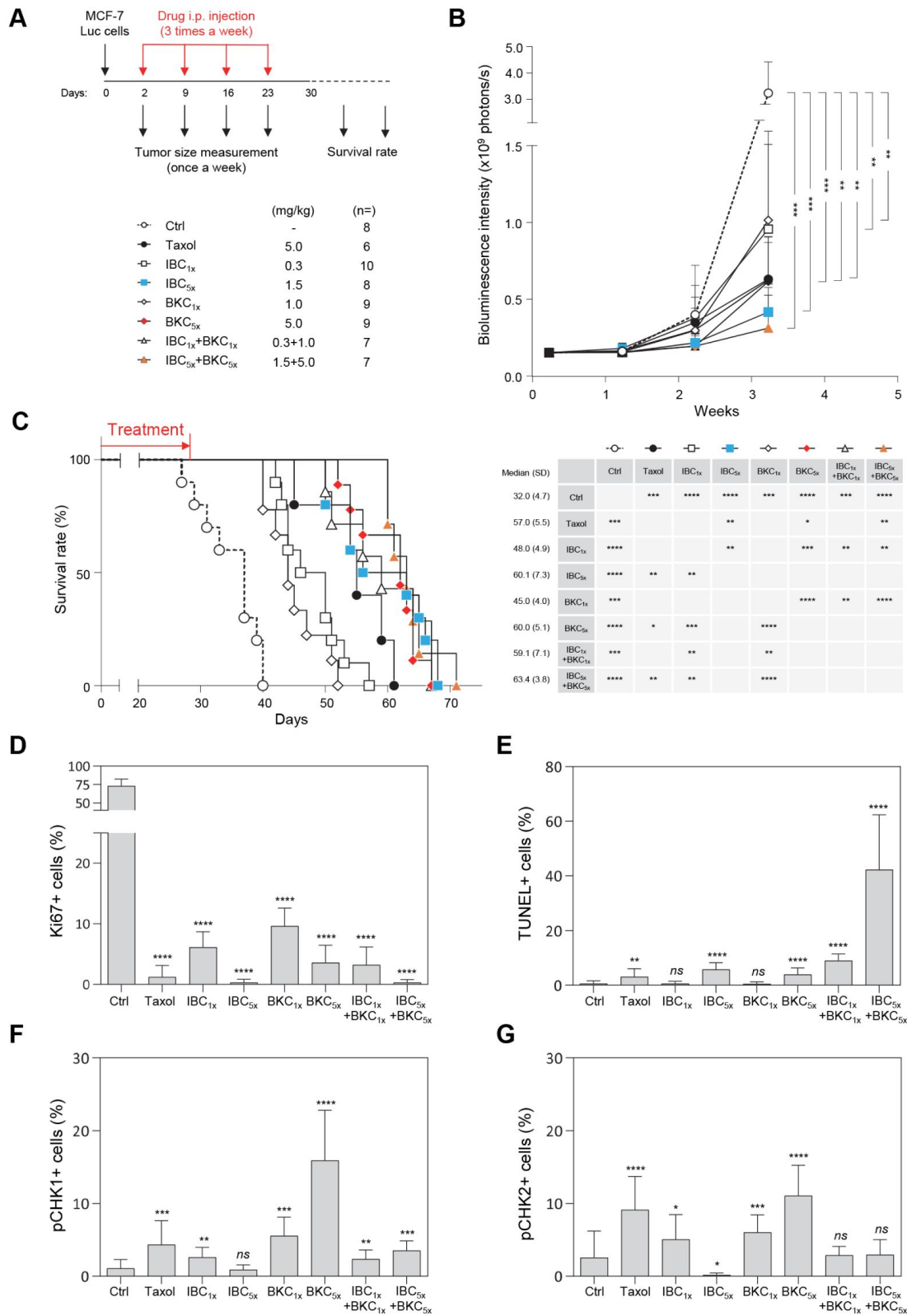


Fig. 6.

IBC and BKC synergistically inhibit tumor development and induce DNA damage in a xenograft mouse model.

A. Schematic description of the protocol. MCF-7/Luc cells (1×10^4) were injected into the fat pads of female NOD/SCID mice at Day 0. Two days later, randomized mice were injected intraperitoneally with PBS, Taxol, IBC, BKC, or IBC+BKC at the indicated doses. Tumor sizes were measured weekly thereafter. Tumor tissues were collected 28 days after grafting and analyzed by immunohistochemistry. Mice survival was also evaluated. **B.** Tumor size in the fat pads was measured once per week by using the IVIS bioluminescence system. The number of xenografted mice receiving each treatment is indicated. ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$, Mann-Whitney rank sum test. **C.** Survival (left) and median survival time (days, right) is shown for xenografted mice receiving each treatment. ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$, Mann-Whitney rank sum test. **D.** Tumor tissues collected from xenograft mice were analyzed immunohistochemically for the cell proliferation marker Ki67; **E.** broken DNA and apoptosis marker, TUNEL staining; **F.** pCHK1 (S345) and **G.** pCHK2 (Thr68). ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; ns, not significant, Mann-Whitney rank sum test.

To determine whether the tumor tissues had increased levels of RS or DNA damage when the mice were treated with IBC and BKC, we used immunohistochemistry to monitor cell proliferation (staining for Ki67) and checkpoint activation (staining for CHK1/CHK2 phosphorylation) and the TUNEL fluorescence assay for DNA fragmentation. Overall, IBC and BKC inhibited tumor cell proliferation (**Fig. 6D** and **Fig. S6B**) and induced DNA fragmentation, indicative of tumor cell death by apoptosis (**Fig. 6E** and **Fig. S6B**). Interestingly, IBC_{5x} alone inhibited cell proliferation as efficiently as the IBC+BKC_{5x} combination and better than Taxol (**Fig. 6B**). However, the percentage of TUNEL-positive cells was much higher in mice treated with IBC+BKC_{5x} than either drug alone (**Fig. 6E**), indicating that IBC and BKC have a strong synergistic effect on the induction of apoptosis and DNA breaks. We also observed a dose-dependent increase in CHK1 phosphorylation in tumor tissues from mice treated with BKC (**Fig. 6F**), which is consistent with our observation above that BKC induced CHK1 activation *in vitro*. Moreover, BKC induced phosphorylation of CHK2 on T68, a marker of DNA damage (**Fig. 6G**). However, this activation of CHK1 and CHK2 mediated by BKC was suppressed by the addition of IBC (**Fig. 6F** and **6G**). Since these checkpoint kinases are important to coordinate DNA repair, this would explain why unreparable DNA breaks accumulate in tumors exposed to the IBC+BKC_{5x} combination. Interestingly, treatment with the higher concentration of IBC alone diminished the level of endogenous phosphorylation of both CHK1 and CHK2, which supports the view that IBC prevents the signaling of endogenous RS and DNA damage in tumors (**Fig. 6F** and **6G**). Altogether, our results show that IBC and BKC act synergistically to inhibit tumor growth *in vivo*, induce DNA fragmentation in the tumor and extend mice survival.

IBC potentiates the effect of chemotherapeutic agents on lymphoma cells

One current strategy for new cancer treatments is to inhibit cell cycle checkpoints at the same time as inducing DNA damage with conventional chemotherapeutic agents, thus driving cells to proliferate in the presence of DNA damage, ultimately resulting in their death. We showed above that IBC inhibits the DNA damage checkpoint kinase CHK2, therefore, we investigated whether IBC might enhance the potency of chemotherapeutic drugs. To this end, we used cell lines from patients with diffuse large B-cell lymphoma (DLBCL); this is the most common lymphoid malignancy in adults, accounting for up to 35% of non-Hodgkin lymphomas. Although DLBCL can be cured in over 60% of patients by using rituximab-based chemotherapy regimens, the remainder develop recurrent or progressive disease that is often fatal (Sarkozy & Coiffier, 2013). New therapeutic approaches are still needed to achieve an effective treatment for these patients with

high risk/refractory DLBCL. Since deregulation of DNA repair pathways in DLBCL cells is associated with a poor outcome (Bret *et al.*, 2015 [DOI](#); Bret *et al.*, 2013 [DOI](#)), we reasoned that IBC could potentiate the effect of agents inducing DNA damage in DLBCL cells.

To address this possibility, we first determined the IC₅₀ of IBC for growth inhibition of a panel of DLBCL cell lines. IC₅₀ ranged from 8–28 μ M (Fig. 7A [DOI](#)), similar to the concentrations we found were effective on the solid cancer cell lines we tested (Fig. 1B [DOI](#) and 1C [DOI](#)). To evaluate whether IBC potentiates the growth inhibitory effect of chemotherapeutic agents, we treated the drug-resistant DLBCL cell line U2932 with various concentrations of including etoposide, doxorubicin or 4-hydroxy-cyclophosphamide in the presence of the IC₂₀ of IBC (4.5 μ M) for 72 hours. We found that IBC substantially enhanced cell growth inhibition by all three of these DNA damaging agents (Fig. 7B [DOI](#)). Moreover, by testing a full-range concentration matrix of drug pairs on cell viability after 72 h treatment, we found that IBC synergistically increased the inhibitory effect of doxorubicin and or 4-hydroxy-cyclophosphamide on U2932 cell proliferation (Fig. 7C [DOI](#) and Fig. S7 [DOI](#)), indicating that IBC is a promising candidate to potentiate the effect of these and potentially other conventional chemotherapeutic agents.

Discussion

Approaches using small molecule inhibitors of cell cycle checkpoint kinases have shown promise as antitumor agents in preclinical studies, either when used alone or in combination with genotoxic agents that induce RS. However, clinical trials with these agents have been disappointing, showing only limited benefit to patients and significant side effects, particularly in combination therapies (Zhu *et al.*, 2020 [DOI](#)). Here, we sought to identify novel small molecule inhibitors that sensitize cancer cells to RS but are not toxic to non-cancer cells. By screening a collection of plant extracts used in traditional Chinese medicine, we identified two known compounds, bakuchiol (BKC) and isobavachalcone (IBC), from *Psoralea corylifolia*, that synergistically inhibit proliferation of cancer cells. We show that BKC inhibits DNA replication, likely by binding to DNA polymerases, and that IBC prevents DNA end resection by inhibiting the checkpoint kinase CHK2. Together, these drugs induce replication stress and impede HR-mediated DSB repair. Consistent with these effects, we show that the drugs act synergistically to inhibit tumor development and extend survival rate in a xenograft mouse model. Moreover, IBC potentiates the inhibitory effect of conventional chemotherapeutic agents on lymphoma cell lines.

BKC is similar in structure to resveratrol and has been reported to exert a variety of pharmacological effects (Xin *et al.*, 2019 [DOI](#)). In particular, BKC inhibits the proliferation of lung, breast, skin and stomach cancer cell lines (Kim *et al.*, 2016 [DOI](#); Li *et al.*, 2016 [DOI](#); Lv & Liu, 2017 [DOI](#)) and prevents replication of the polyomavirus SV40 *in vitro* (Sun *et al.*, 1998 [DOI](#)). This is consistent with our evidence from *in silico* molecular docking that BKC binds the catalytic site of DNA polymerases δ and ϵ . Thus, BKC probably inhibits the polymerases by competing for dNTP binding. Indeed, we demonstrate that BKC inhibits replication elongation of chromosomal DNA and ssDNA in *Xenopus* egg extracts, albeit to a lesser extent than does aphidicolin. These findings argue against the possible explanation that BKC inhibits dNTP synthesis, as proposed for the structurally similar compound resveratrol (Benslimane *et al.*, 2020 [DOI](#); Fontecave *et al.*, 1998 [DOI](#)), since dNTPs are available in large amount in these extracts. Moreover, we show that BKC slows replication fork progression *in vivo* in both normal and cancer cells, indicating that it acts as a *bona fide* replication inhibitor.

IBC affects a wide spectrum of biological functions (Kuate & Sandjo, 2012 [DOI](#)), including the activity of the NAD⁺-dependent deacetylase Sirtuin 2 (Ren *et al.*, 2024 [DOI](#)) and shows antitumor activity against drug-resistant cancers (Kuate *et al.*, 2015 [DOI](#); Ren *et al.*, 2024 [DOI](#); Wu *et al.*, 2022 [DOI](#)). IBC is thought to prevent cell proliferation and induce apoptosis in various cancer cell models by inhibiting the AKT kinase (Jin & Shi, 2016 [DOI](#); Jing *et al.*, 2010 [DOI](#)). In our hands, however, IBC does

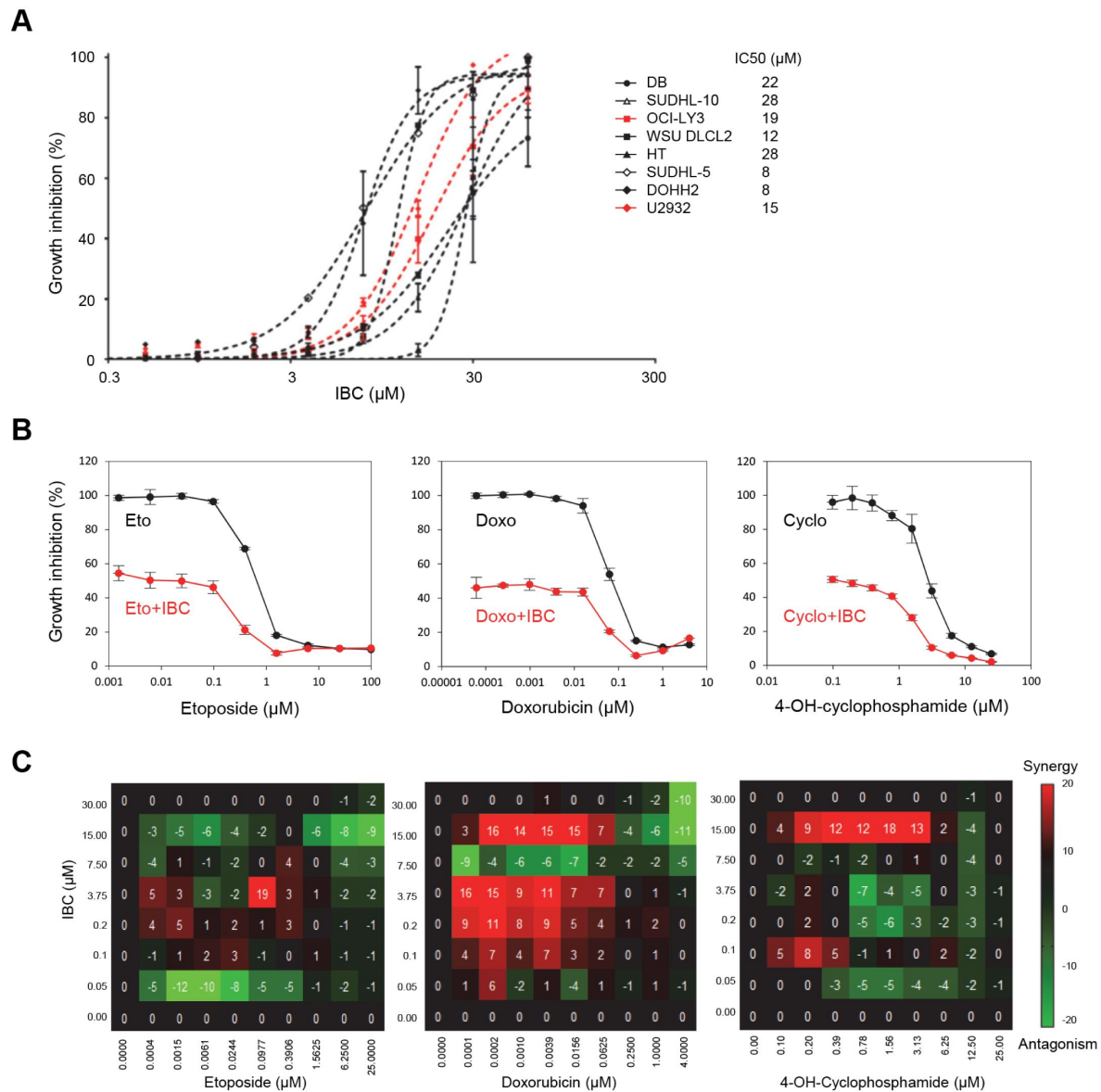


Fig. 7.

IBC potentiates the effect of chemotherapeutic agents on lymphoma cells.

A. A panel of eight diffuse large B-cell lymphoma (DLBCL) cell lines were incubated with the indicated concentrations of IBC for 72 hours (left) and growth inhibition was measured to calculate the IC₅₀ in each cell line (right). Data are presented as means $\pm$ SD (n=3). **B.** U2932 cells were treated with the indicated concentrations of etoposide (Eto), doxorubicin (Doxo) or 4-OH-cyclophosphamide (4-OH-Cyclo) without (black points) or with 1.5 $\mu\text{g}/\text{ml}$ (the IC₂₀ concentration for this cell line) IBC (red points) for 72 hours. Cell viability was measured. Data are presented means $\pm$ SD (n=3). **C.** Full-concentration matrix analyses of U2932 cells treated with IBC and Eto (left), Doxo (middle) or 4-OH-Cyclo at the indicated concentrations for 72 hours. Synergy and antagonism were calculated as described in Fig. 1.

not significantly inhibit AKT, at least when the drug was used at concentrations that inhibit cell proliferation. Rather, IBC inhibits CHK2 *in vivo* and *in vitro* at concentrations an order of magnitude lower than those that inhibit other kinases and it has no effect on the activity of the related checkpoint kinase CHK1. As a key effector of the DNA damage response, CHK2 is an attractive target for new drugs that might potentiate the effect of conventional, DNA damaging treatments for cancer (Bucher & Britten, 2008 [↗](#)). However, few CHK2-specific inhibitors are available and those that are have only modest anti-proliferative effects when compared with inhibitors of other checkpoint kinases such as CHK1 (Ronco *et al.*, 2017 [↗](#)). The results of clinical trials combining the CHK2 inhibitor PHI-101 with other therapeutic agents are still awaited (Park *et al.*, 2022 [↗](#)). Interestingly, CHK2 inhibitors also protects healthy tissues from radiotherapy or chemotherapy, presumably by inhibiting p53-dependent apoptosis (Jiang *et al.*, 2009 [↗](#); Xu *et al.*, 2021 [↗](#)).

The target of IBC *in vivo* is very likely CHK2-dependent activation of BRCA1. Indeed, we show that IBC prevents HR-mediated DSB repair by inhibiting the formation of RAD51 foci in cancer cells. The loading of RAD51 depends on the resection of DNA ends by nucleases, through a process that is mediated by the CHK2-dependent activation of BRCA1 (Parameswaran *et al.*, 2015 [↗](#); Zhang *et al.*, 2004 [↗](#)). We found that IBC inhibits resection at DSBs as efficiently as the CHK2 inhibitor BML-277. In contrast, IBC does not inhibit resection of nascent DNA at stalled forks, a process that does not depend on BRCA1 but, rather, is repressed by BRCA1 and BRCA2 (Chen *et al.*, 2018 [↗](#)). Our data indicate therefore that IBC differentially affects resection at DSB and at stalled forks by inhibiting CHK2.

The inhibitory effects of BKC on DNA synthesis and IBC on DNA end resection support a model in which BKC increases RS in cancer cells by inhibiting bulk DNA synthesis, so increasing the rate of replication fork arrest/collapse and inducing RS-dependent DSBs, and IBC inhibits HR-mediated DSB repair, resulting in cell death by apoptosis (**Fig. 8** [↗](#)). This model explains why BKC and IBC synergistically inhibit the proliferation of cancer cells: BKC-mediated RS adds to oncogene-induced RS, explaining the differential sensitivity of normal and cancer cells to the combination of BKC and IBC, and also to the effects of IBC alone.

In conclusion, our screening of traditional herbal medicine for novel combinations of small molecule inhibitors identified two compounds, BKC and IBC, acting synergistically to block cancer cell growth in different *in vitro* and *in vivo* contexts. Although it is unlikely that these compounds will ever be used in combination therapies, our results indicate that IBC or other clinically approved CHK2 inhibitors, could be used to potentiate the cytotoxic effect of known DNA damaging agents. This view is supported by our data showing that IBC potentiates the effect of etoposide, doxorubicin or 4-hydroxy-cyclophosphamide on drug-resistant lymphoma cell lines. Finally, recent evidence indicates that RS and DNA damage stimulate anti-tumor immunity by activating cytosolic nucleic acid-sensing pathways (Chabanon *et al.*, 2021 [↗](#); Tarsounas & Sung, 2020 [↗](#); Técher & Pasero, 2021 [↗](#)). New combinations of genotoxic agents and inhibitors targeting RS checkpoint kinases might thus be valuable also for potentiating immune checkpoint inhibitors.

Materials and Methods

Cell Culture

Immortalized human BJ fibroblasts from Dr. D. Peeper (The Netherlands Cancer Institute, Amsterdam) and MCF-7/Luc were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 100 U/ml penicillin/streptomycin. SUM159 cells were obtained from Asterand Bioscience, UK, and grown in Ham's F-12 medium supplemented with 5% fetal calf serum, 10 µg/ml insulin, 1 µg/ml hydrocortisone, 100 µg/ml streptomycin and 100 U/ml penicillin. MCF-7, A549, HCC827, PC3, DU145, U937, HCT116 and OVCAR8 cancer cells were

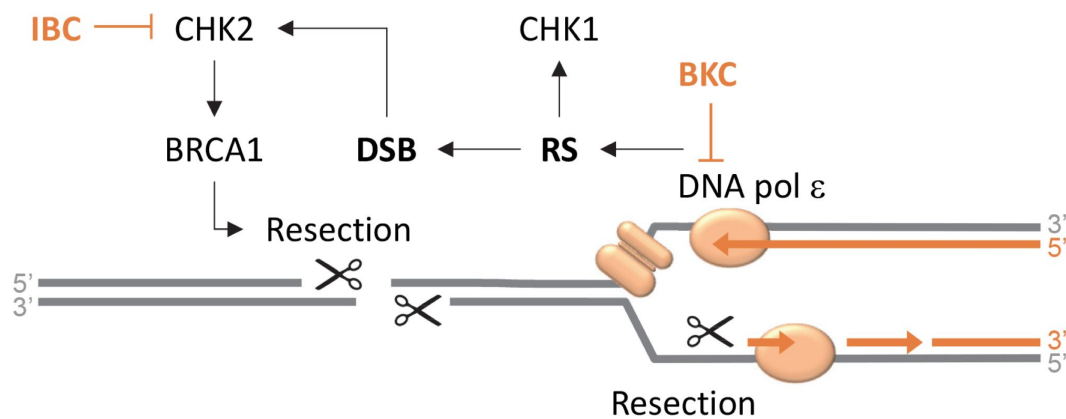


Fig. 8.

Model of the synergistic action of BKC and IBC that kills cancer cells.

By inhibiting the DNA polymerases, BKC enhances the endogenous RS in cancer cells, leading to increased DNA damage. IBC specifically inhibits CHK2 to prevent DNA repair. The combined use of BKC and IBC enhances DNA damage to a level that triggers cancer cell death.

purchased from ATCC and cultured in DMEM (MCF-7, A549, DU145, and OVCAR8) or RPMI-1640 (HCC827, PC3, U937, HCT116) supplemented with 10% fetal calf serum and 100 U/ml penicillin/streptomycin at 37°C in 5% CO₂. DLBCL cell lines (DB, SUDHL-10, OCI-LY3, WSU DLCL2, HT, SUDHL-5, SOHH2 and U292) were cultures in RMPI-1640 supplemented with 10% fetal calf serum and 100 U/ml penicillin/streptomycin at 37°C in 5% CO₂.

Reagents

Isobavachalcone was purchased from Sigma-Aldrich (SML1450) or Abcam (ab141168). Bakuchiol was from Abcam (ab141036). Cell proliferation reagent WST-1 was from Sigma-Aldrich (5015944001).

Pulse-field gel electrophoresis (PFGE)

Subconfluent cultures (10-cm plates) were treated as specified. Cells were harvested by trypsinization, and plugs of 2% (w/v) agarose containing 0.5×10^6 cells in PBS were prepared using a CHEF disposable plug mold (Bio-Rad). The plugs were incubated in lysis buffer (100 mM EDTA, 1% (w/v) sodium lauryl sarcosinate, 0.2% (w/v) sodium deoxycholate, 1 mg/ml proteinase K) at 37 °C for 24 hours and were then washed with washing buffer (20 mM Tris pH8, 50 mM EDTA pH8). Pulsed-field gel electrophoresis was carried out at 13 °C for 23 hours in 0.9% (w/v) agarose containing 0.25% TBE buffer using a Biometra Rotaphor (Biometra). The parameters were as follows: voltage 180–120 V log; angle from 120° to 110° linear; interval 30–5 s log. The gel was stained with ethidium bromide (EtBr) and analyzed using ImageJ.

Flow cytometry analysis

Cells were pulse labeled with 10 μM EdU for 30 minutes. After fixation with 1% formaldehyde for 30 minutes at room temperature and permeabilized in 0.25% Triton X-100 for 15 minutes, EdU incorporation was detected by using Click chemistry according to the manufacturer's instructions (Click-iT EdU Flow Cytometry Cell Proliferation Assay, Invitrogen). The cells were resuspended in PBS containing 1% (w/v) BSA, 2 μg/ml DAPI and 0.5 mg/ml RNase A for 30 min at room temperature and were analyzed in a MACSQuant flow cytometer (Miltenyi Biotec). The percentages of cells in G₁, S and G₂/M phases were quantified by using FlowJo single-cell analysis software (FlowJo, LLC).

DNA fiber spreading

DNA fiber spreading was performed as described previously (Coquel *et al*, 2018 [Coquel et al, 2018](#); Jackson & Pombo, 1998 [Jackson & Pombo, 1998](#)). Briefly, subconfluent cells were sequentially labeled first with 10 μM 5-iodo-2'-deoxyuridine (IdU) and then with 100 μM 5-chloro-2'-deoxyuridine (CldU) for the indicated times. One thousand cells were loaded onto a glass slide (StarFrost) and lysed with spreading buffer (200 mM Tris-HCl pH 7.5, 50 mM EDTA, 0.5% SDS) by gently stirring with a pipette tip. The slides were tilted slightly and the surface tension of the drops was disrupted with a pipette tip. The drops were allowed to run down the slides slowly, then air dried, fixed in methanol/acetic acid 3:1 for 10 minutes, and allowed to dry. Glass slides were processed for immunostaining with mouse anti-BrdU to detect IdU, rat anti-BrdU to detect CldU, mouse anti-ssDNA antibodies (see Supplemental Information for details) and corresponding secondary antibodies conjugated to various Alexa Fluor dyes. Nascent DNA fibers were visualized by using immunofluorescence microscopy (Leica DM6000 or Zeiss ApoTome). The acquired DNA fiber images were analyzed by using MetaMorph Microscopy Automation and Image Analysis Software (Molecular Devices) and statistical analysis was performed with GraphPad Prism (GraphPad Software). The lengths of at least 150 IdU and/or CldU tracks were measured per sample.

Single-molecule analysis of resection tracks (SMART)

Cells were labeled with 10 μ M BrdU for 24 hours. They were then treated with 5 μ M bleomycin (Calbiochem) for 1 h and harvested at the indicated time points. They were processed for DNA fiber spreading as described (Altieri *et al.*, 2020 [↗](#); Cruz-García *et al.*, 2014 [↗](#)). BrdU tracks were stained with anti-BrdU antibody without DNA denaturation and visualized by fluorescence microscopy (Zeiss ApoTome). The acquired DNA fiber images were analyzed by using MetaMorph Microscopy Automation and Image Analysis Software (Molecular Devices) and statistical analysis was performed with GraphPad Prism (GraphPad Software). The lengths of at least 200 BrdU tracks were measured per sample.

Cellular Thermal Shift Assay (CETSA)

CETSA was performed according to the protocol described by Delport and Hewer (Delport & Hewer, 2022 [↗](#)) with the following modifications. MCF-7 cells were treated with DMSO, IBC, or BML-277 for 2 hours, then trypsinized and pelleted. Cells were resuspended in PBS and evenly distributed into different Eppendorf tubes at 10^6 cells per tube. Tubes were incubated at specified temperatures as indicated for 3 minutes, cooled down at 25°C for 3 minutes and then incubated on ice for another 3 minutes. Cells were pelleted down by centrifugation. The pellets were then lysed with RIPA buffer in the presence of benzonase for 20 minutes at 4°C before further centrifugation. The supernatant was collected and subjected to SDS-PAGE before western blotting for the detection of CHK1 and CHK2.

RPA foci detection

For the detection of chromatin-bound RPA foci, cells seeded on the coverslips were fixed with 4% PFA in PBS for 15 minutes and then incubated for 3 minutes at 4 °C with CSK buffer (10 mM PIPES pH 6.8, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 300 mM sucrose, 0.5 mM DTT) containing 0.25% Triton X-100 and phosphatase inhibitor cocktail (Sigma- Aldrich, P0044). The coverslips were incubated with an anti-RPA antibody (overnight at 4 °C) and then with a secondary antibody conjugated to an Alexa Fluor dye for 1 hour at 37 °C, followed by DAPI staining. Images were acquired by using a Zeiss ApoTome microscope. The mean fluorescence intensity in EdU-positive cells was quantified by using CellProfiler (<http://www.cellprofiler.org> [↗](#)).

γ -H2AX foci detection

Cells seeded on coverslips were treated and labeled with 10 μ M EdU for 10 minutes as described. They were washed twice with PBS. They were fixed in fixation buffer (2% PFA) for 10 minutes at room temperature and permeabilized in permeabilization buffer (0.1% Na Citrate, 0.1% Triton X-100). The coverslips were incubated with an anti- γ -H2AX antibody overnight at 4°C after blocking in PBS containing 1% BSA in PBS for 1 h at room temperature. Coverslips were incubated with a secondary antibody conjugated to Alexa Fluor dye, followed by Click chemistry reaction and DAPI staining. Images were acquired using a Zeiss ApoTome microscope. The mean fluorescence intensity (MFI) in EdU-positive cells was quantified using CellProfiler (<http://www.cellprofiler.org> [↗](#)).

DNA end resection assay

Measure of resection was performed as described previously with the following modifications (Zhou *et al.*, 2014 [↗](#)). Genomic DNA was extracted from fresh cells using the QIAamp mini kit (Qiagen). 500 ng DNA was then treated with 5 units of RNase H. 200 ng RNase H-treated DNA were digested or not with the Ban I restriction enzyme (16U per sample) overnight at 37 °C, which cuts at ~200 bp from the DSB-KDEL3 and at 740 bp for DSB-ASXL1. Ban1 was heat inactivated 20 min at 65 °C. Digested and undigested DNA were analyzed by qPCR using the following primers:

DSB-KDEL3_200 FW: ACCATGAACGTGTTCCGAAT;

DSB-KDEL3_200_REV: GAGCTCCGCAAAGTTTCAAG;

DSB-ASXL1_740 FW: GTCCCCTCCCCACTATTT;

DSB-ASXL1_740_REV: ACGCACCTGGTTTAGATTGG;

ssDNA% was calculated with the following equation: $\text{ssDNA\%} = 1/(2^{(\text{Ct digested} - \text{Ct undigested} - 1)} + 0.5) * 100$.

In vitro kinase assay

Kinase selectivity was evaluated using KinaseProfiler service provided by Eurofins. <https://www.eurofinsdiscoveryservices.com/cms/cms-content/services/in-vitro-assays/kinases/kinase-profiler/>

DNA replication assay using *Xenopus* egg extracts

Cytoplasmic extracts (low speed and high speed) and demembranated sperm nuclei were prepared as previously described (Mechali & Harland, 1982 [\[1\]](#); Murray, 1991 [\[2\]](#)), snap frozen in liquid nitrogen and stored at -80 °C. Upon thawing, extracts were supplemented with cycloheximide (250 µg/ml) and an energy regeneration system (1 mM ATP, 2 mM MgCl₂, 10 µg/ml creatine kinase, 10 mM creatine phosphate).

Egg extracts were supplemented with α-[32P] dATP (3000 Ci/mmol, Perkin Elmer) and either demembranated sperm nuclei (1000/l of extract) or M13 ssDNA (200 ng/µl; NEB). At the indicated time points, samples were neutralized in 10 mM EDTA, 0.5% SDS, 200 µg/ml Proteinase K (Sigma) and incubated at 37°C overnight. Incorporation of radioactive label was determined by TCA precipitation on GF/C glass fiber filters (Whatman) following by scintillation counting.

Full-range dose matrix approach

To investigate the interactions between two-drug combinations, we used a synergy matrix assay that was previously described in details (Tosi *et al.*, 2018 [\[3\]](#)). The effects of drug combinations on cell growth was evaluated by standard sulforhodamine B (SRB) assay as described (Orellana & Kasinski, 2016 [\[4\]](#)). Briefly, exponentially growing cells were treated with all the combinations of 5 concentrations of BKC and 8 concentrations of IBC in 96-well plates for 72h. Cells were then fixed with trichloroacetic acid solution (10%) and stained with a 0.4% sulforhodamine B solution in 1% acetic acid, washed with 1% acetic acid and incubated with 10 mM Tris-HCl solution for 10 min with gentle shaking. Absorbance at 560 nm was then measured using a PHERAstar FS plate reader (BMG Labtech, Ortenberg, Germany) and cell survival (blue matrix) was calculated in comparison with untreated cells. Experiments have been performed three times independently for each cell line and a representative matrix is shown as an example for each cell line. A synergy matrix was then calculated as described previously (Tosi *et al.*, 2018 [\[3\]](#)) to quantify the interaction effect: a red color in the matrix indicates a synergism, a black color additivity and a green color an antagonism.

In silico molecular docking

To explore human DNA polymerase and BKC interaction, BKC was docked into the polymerase active site using GOLD docking tool with BIOVIA Discovery Studio (Dassault Systèmes, BIOVIA Corp., San Diego, CA, USA). In order to construct human polymerase delta and epsilon protein models, we carried out homology modeling using yeast polymerase delta (PDB code 3IAY) and epsilon from *Saccharomyces cerevisiae* (PDB code 4M8O) as templates, respectively. Similarly, to investigate the interaction between checkpoint kinase and IBC, IBC was docked into the active site

of CHK1 (PDB code 5F4N) and CHK2 (PDB code 4BDK). The crystal structures of DNA polymerase and checkpoint kinase were downloaded from RCSB Protein Data Bank. The proteins and compound atoms were applied with CHARMM force field.

Animal study

Female NOD/SCID mice were National Laboratory Animal Center, Taiwan. All mice had free access to chow and water, and were housed at 21-23°C with 12 h light-12 h dark cycles. All mice were handled in accordance with the guidelines laid out by the Academia Sinica Institutional Animal Care and Utilization Committee (Protocol No. 10-12-097). To generate MCF tumor-bearing mice, MCF-7/Luc cells (1×10^4) were subcutaneously inoculated into the fat pad of the mice as published (Kuo *et al.*, 2017 [\[1\]](#)). Eight groups of the mice received an intraperitoneal injection of PBS (Ctl), Taxol (5 mg/kg), IBC (0.3 and 1.5 mg/kg), BKC (1 and 5 mg/kg), and a combination of IBC and BKC (0.3 mg/kg IBC + 1 mg/kg BKC and 1.5 mg/kg IBC + 5 mg/kg BKC), thrice a week, from day 2 to 30 after tumor graft. The mice were daily measured for survival rate. Their tumor growth was weekly monitored using the IVIS system (Xenogen, USA). The signal of the bioluminescence from mice was quantified using Living Image 2.5 (Xenogen) as photons/sec/region of interest (ROI).

Immunohistochemical (IHC) analysis

Tumors were removed from mice 28 days post tumor inoculation. The tumors were fixed, dehydrated and embedded into paraffin. The tumor sections were stained with the antibody against Ki67, p-CHK1, and p-CHK2 and TUNEL kits. The signal of the sections was visualized and quantified by AxioVision software (Carl Zeiss MicroImaging).

Antibodies

Antibody	Catalog No.	Dilution
Mouse anti-BrdU clone B44	347580, BD Biosciences	1/100
Rat anti-BrdU clone BU1/75	ABC117-7513, Eurobio Abcys	1/100
Anti-ssDNA	MAB3868, Millipore	1/250
Anti-pCHK1 (S345)	2348, Cell signaling	1/1000
Anti-pCHK2 (T68)	2661, Cell signaling	1/1000
Anti- γ -H2AX (S139)	05-636, Millipore	1/500
Anti-actin	A4700, Sigma	1/500
Anti-RPA1	Ab79398, Abcam	1/300
Anti-TBP	8515, Cell Signaling	1/1000
Anti-CHK1	2360, Cell Signaling	1/1000
Anti-pRPA32 (S4/S8)	A300-245A, Bethyl	1/1000
Anti-CHK2	ab109413, Abcam	1/5000
Anti-RPA2	A303-874, Bethyl	1/500
Anti-pCHK2 (S516)	2669, Cell signaling	1/1000
Anti-pChk1 (S296)	2349, Cell signaling	1/1000
Anti-pBRCA1 (S988)	166793, Cell Signaling	1/200
Anti-RAD51	PC130, Millipore	1/500
Anti-pAKT (S473)	9271, Cell signaling	1/1000

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Declarations

Ethical Approval

All mice were handled in accordance with the guidelines laid out by the Academia Sinica Institutional Animal Care and Utilization Committee (Protocol No. 10-12-097).

Competing interests

The authors declare no conflict of interest.

Authors' Contribution

WCY, YLL and PPa conceived and planned the study. FC, AB and YLL performed the experiments on replication stress and the DNA damage response. KCT performed *in silico* molecular docking. JD and JM performed the experiments on DLBCL cells. MKH and PPo performed the experiments and analyses on drug synergy. DM designed, supervised and analyzed the experiments with *Xenopus* egg extracts. SZH, THC and WCY designed and performed the animal experiments. YLL and PPa wrote the manuscript and all authors reviewed it.

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Availability of data and materials

Not applicable

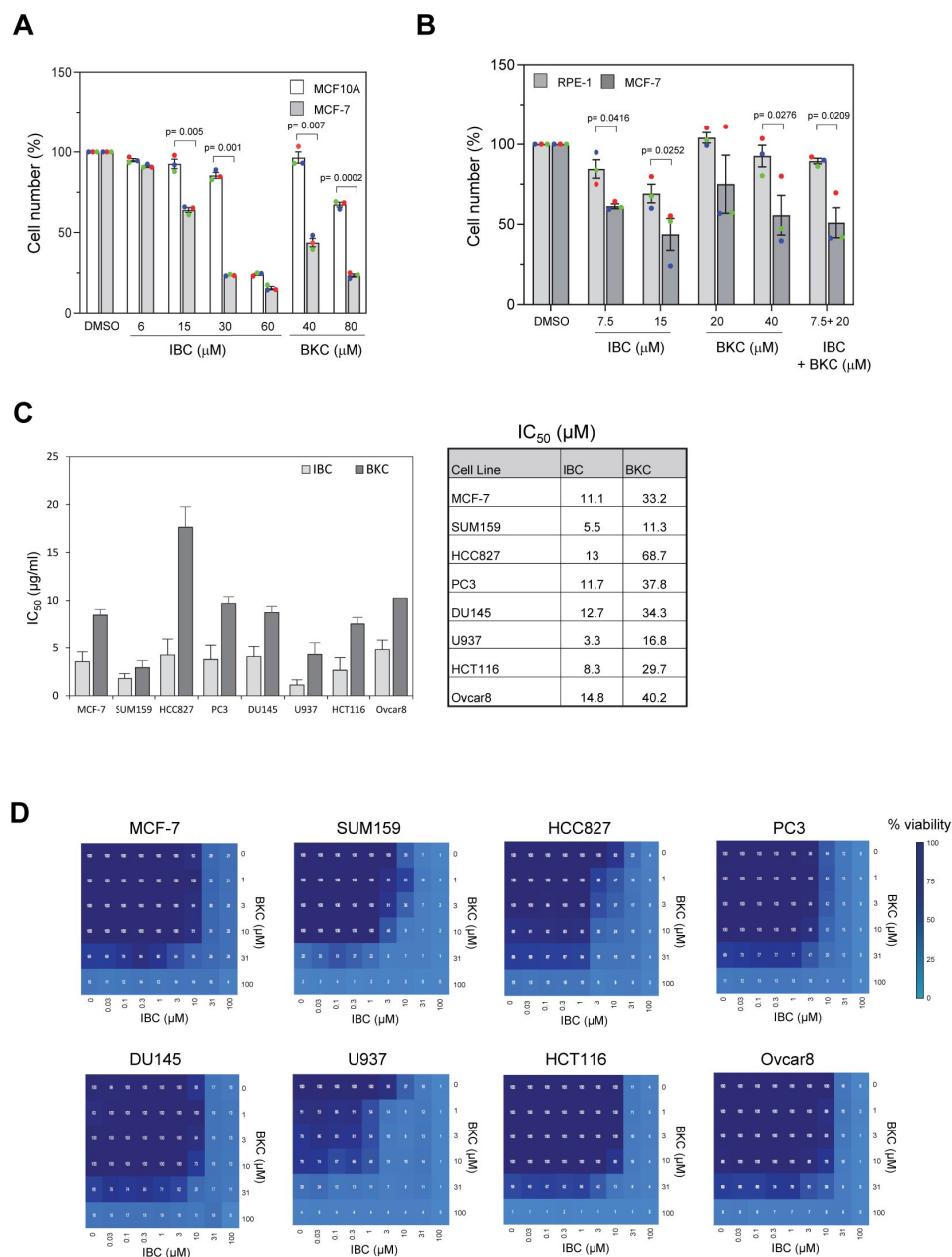


Fig. S1.

IBC and BKC synergistically inhibit proliferation of cancer cell lines.

A. MCF10A and MCF-7 cells were treated with DMSO, or indicated concentrations of IBC and BKC for 72 hours. Cell number was quantified by using the WST-1 assay. Mean $\pm$ SD of three independent experiments are shown. The p-values were calculated by two-tailed unpaired t-test. **B.** Human telomerase-immortalized RPE-1 cells and MCF-7 and A549 cells were treated with DMSO, indicated concentrations of IBC or BKC or the combination 7.5 μ M IBC and 20 μ M BKC for 72 hours. Cell number was quantified by using the WST-1 assay. Mean $\pm$ SD of three independent experiments are shown. The p-values were calculated by two-tailed unpaired t-test. **C.** A panel of cancer cell lines were treated with different concentrations of IBC or BKC for 72 hours. Cell viability was measured and IC_{50} for each cell line was calculated from 3 independent experiments. Mean $\pm$ SD is shown. **D.** Viability matrix for the concentrations of IBC and BKC combinations tested in a panel of cancer cell lines. Percentage of cell viability is indicated by the blue gradient. A representative analysis of three independent experiments is shown.

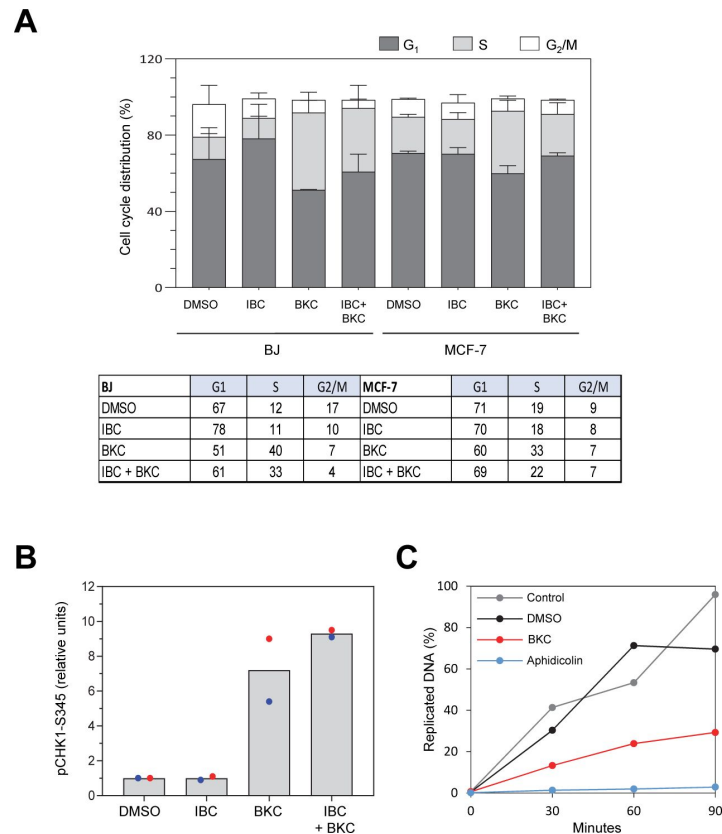


Fig. S2.

BKC inhibits DNA replication and induces S-phase accumulation in the cell cycle.

A. BJ and MCF-7 cells were treated for 24 hours with IBC, BKC or the combination IBC + BKC for 24 hours, then labelled with 10 μ M EdU for 30 minutes. Cell cycle distribution was analyzed by flow cytometry. Data are presented as mean $\pm$ SD from three independent experiments. **B.** Densitometric quantification of the pCHK1-S345 signal for **Fig. 2C**. **C.** *Xenopus* High Speed (HSS) egg extracts were treated with DMSO, Bakuchiol (BKC, 100 μ M, 25 μ g/ml) or aphidicolin (Aph, 60 μ M) before the addition of ssDNA. Samples were collected at 30, 60 or 90 minutes after the addition of sperm DNA. The percentage of replicated DNA was calculated as described in materials and methods. Representative figure from two independent experiments is shown.

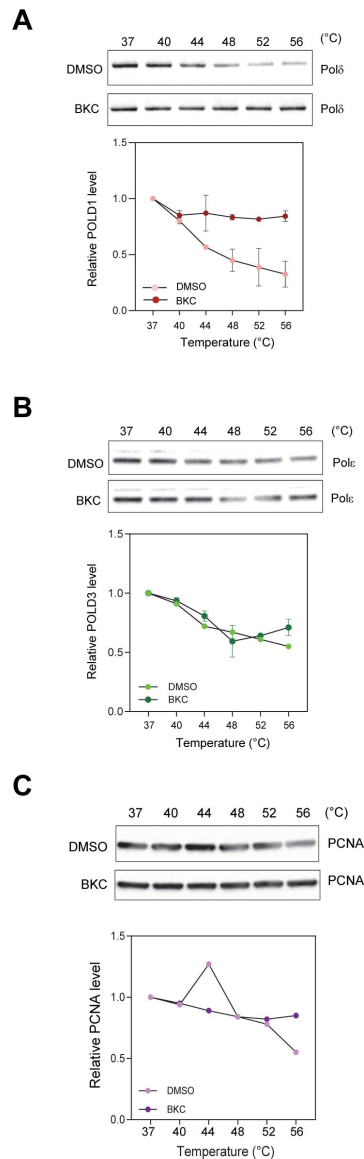


Fig. S3.

BKC does not interact with PCNA.

A-C. *Xenopus* egg extracts were incubated with DMSO or 40 μ M BKC for 2 hours prior to the cellular thermal sensitivity shift assay (CETSA) at indicated temperature, as described in the Materials and Methods. The residual amount of the DNA Pol δ catalytic subunit POLD1 (p125, **A**) and the accessory subunit POLD3 (p66, **B**) or PCNA (**C**) in the supernatant was detected by western blotting. Mean of two independent experiments is shown.

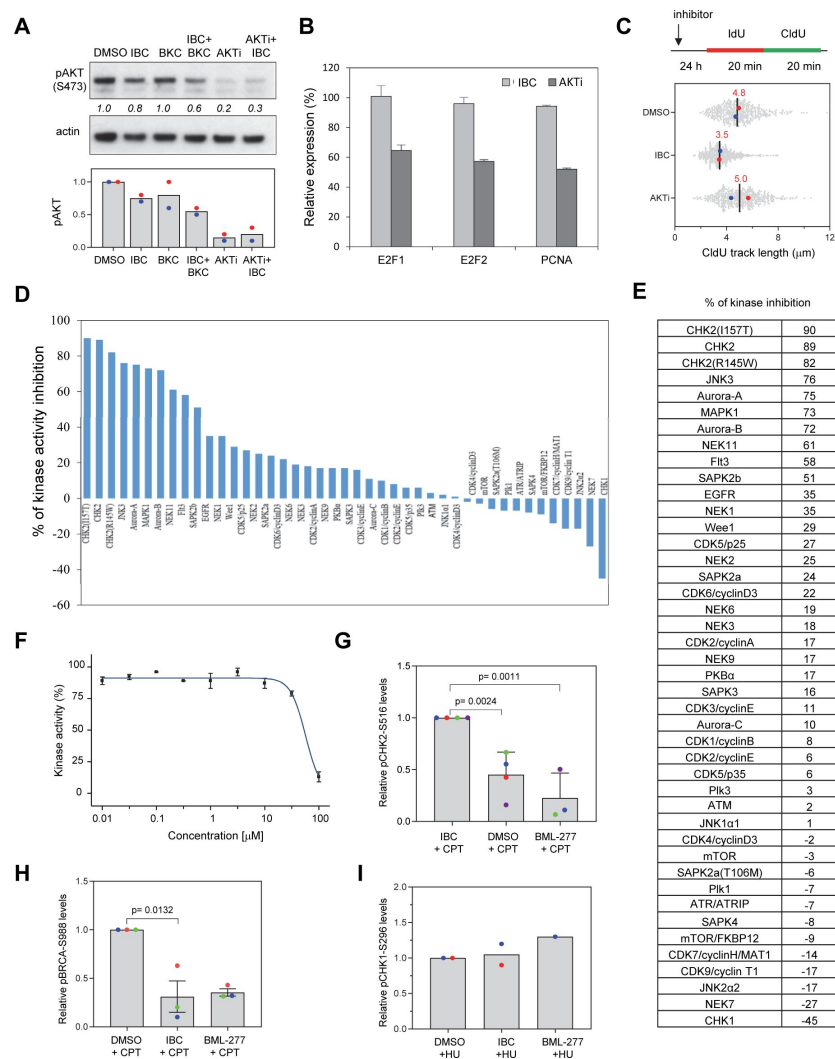


Fig. S4.

IBC does not inhibit AKT activity in MCF-7 cells.

A. MCF-7 cells treated with DMSO, 15 μ M IBC, 20 μ M BKC, the combination IBC + BKC or AKT inhibitor, MK-2206, (AKTi, 10 μ M) for 24 hours. Auto-phosphorylation of AKT was detected by immunoblotting analysis. Densitometric quantification of phosphor-AKT signal is shown. (n=2) **B.** MCF-7 cells treated overnight with DMSO, IBC or AKT inhibitor (AKTi). Total RNA was isolated. Reverse transcription and quantitative PCR was performed with specific primers targeting the gene bodies of PCNA, E2F1 and E2F2. (n=2) **C.** MCF-7 cells were treated with IBC or AKT inhibitor (AKTi) for 24h. They were then sequentially labelled with IdU and CldU for 20 minutes. Replication fork progression was measured using DNA fiber spreading. The median length of CldU tracks of two biological replicates is indicated in red. **D.** *In vitro* kinase assay of 43 cell cycle-related kinases following treatment with 30 μ M IBC. The CHK2(I157T) mutation is linked to an increased risk of breast and colorectal cancers. CHK2(R145W) is associated with Li-Fraumeni Syndrome. Both mutations do not affect the basal kinase activity of CHK2. **E.** Percentage inhibition of kinase activity by IBC treatment is shown. **F.** AKT kinase peptides were incubated with a half-log range dilution series of Isobavachalcone and *in-vitro* kinase activity was measured using a radiometric assay. Data are presented as mean $\pm$ SD with a technical triplicate. **G.** Densitometric quantification of the pCHK2-S516 induction by CPT for **Fig. 4B**. The relative induction of pCHK2-S516 by CPT after IBC or BML-277 treatment compared to DMSO control is indicated. Mean $\pm$ SD of 3 independent experiments for DMSO+CPT and IBC+CPT are shown. The p-value was determined using two-tailed unpaired t-test. **H.** Densitometric quantification of the pCHK2-S516 induction by CPT for **Fig. 4C**. The relative induction of pBRCA1-S988 by CPT after IBC or BML-277 treatment compared to DMSO control is indicated. Mean $\pm$ SD of 3 independent experiments for DMSO+CPT and IBC+CPT are shown. The p-value was determined using two-tailed unpaired t-test. **I.** Densitometric quantification of the pCHK1-S296 induction by HU for **Fig. 3D**. The relative induction of pCHK1-S296 by HU after IBC or BML-277 treatment compared to DMSO control is indicated (n=2).

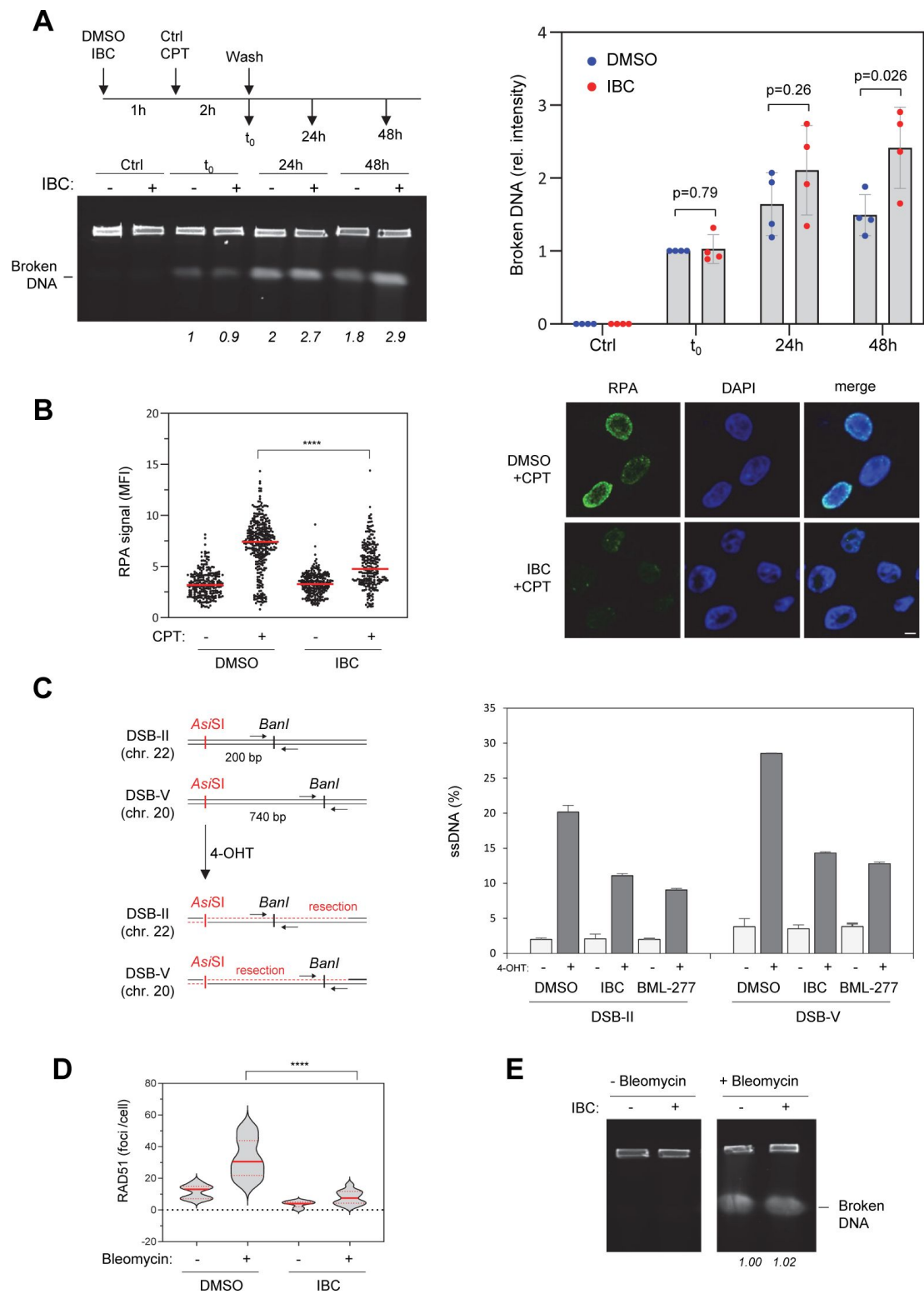


Fig. S5.

IBC results in the persistence of DNA breaks and impairs DNA end resection.

A. MCF-7 cells were pre-treated with DMSO or IBC for 1 hour, followed by incubation with or without camptothecin (CPT, 1 μ M) for another 2 hours. Cells were recovered immediately or washed and let recover in the presence of DMSO or IBC for 24 or 48 hours. The amount of broken DNA was detected by PFGE. Fold increase of broken DNA was normalized by the control sample at t0. The *p*-values are indicated (two-tailed paired *t*-test). Representative gel image from 4 independent experiments is shown. **B.** MCF-7 cells were pre-treated with DMSO or IBC for 1 hours, followed by incubation with or without camptothecin (CPT) for another 2 hours. Cells were fixed and the formation of RPA foci was detected by CSK-immunofluorescence microscopy. Mean fluorescence intensity (MFI) of RPA signal was quantified by CellProfiler. **** $p < 0.0001$, Mann-Whitney rank sum test. Representative data from 3 independent experiments are shown. Bar: 5 μ m. **C.** D1A cells were treated with either DMSO, IBC or CHK2 inhibitor (BML-277) for 2 hours before induction of DNA breaks by 4-hydroxytamoxifen (4-OHT) for 4 hours. Resection at two break sites is evaluated. Percentage of ssDNA was calculated as indicated in the Material and Methods. **D.** MCF-7 cells were pre-treated with DMSO, IBC for 2 hours, followed by bleomycin treatment for 1 hour. Cells were allowed to recover for 1 hour and the formation of RAD51 foci was detected by CSK-immunofluorescence microscopy. RAD51 foci number was quantified using CellProfiler. **** $p < 0.0001$, Mann-Whitney rank sum test. A representative example of three independent experiments is shown ($n=3$). **E.** MCF-7 cells were pre-treated with DMSO, IBC for 2 hours, followed by bleomycin treatment for one hour. The amount of broken DNA was detected by PFGE. Fold increase of broken DNA was normalized by the control sample.

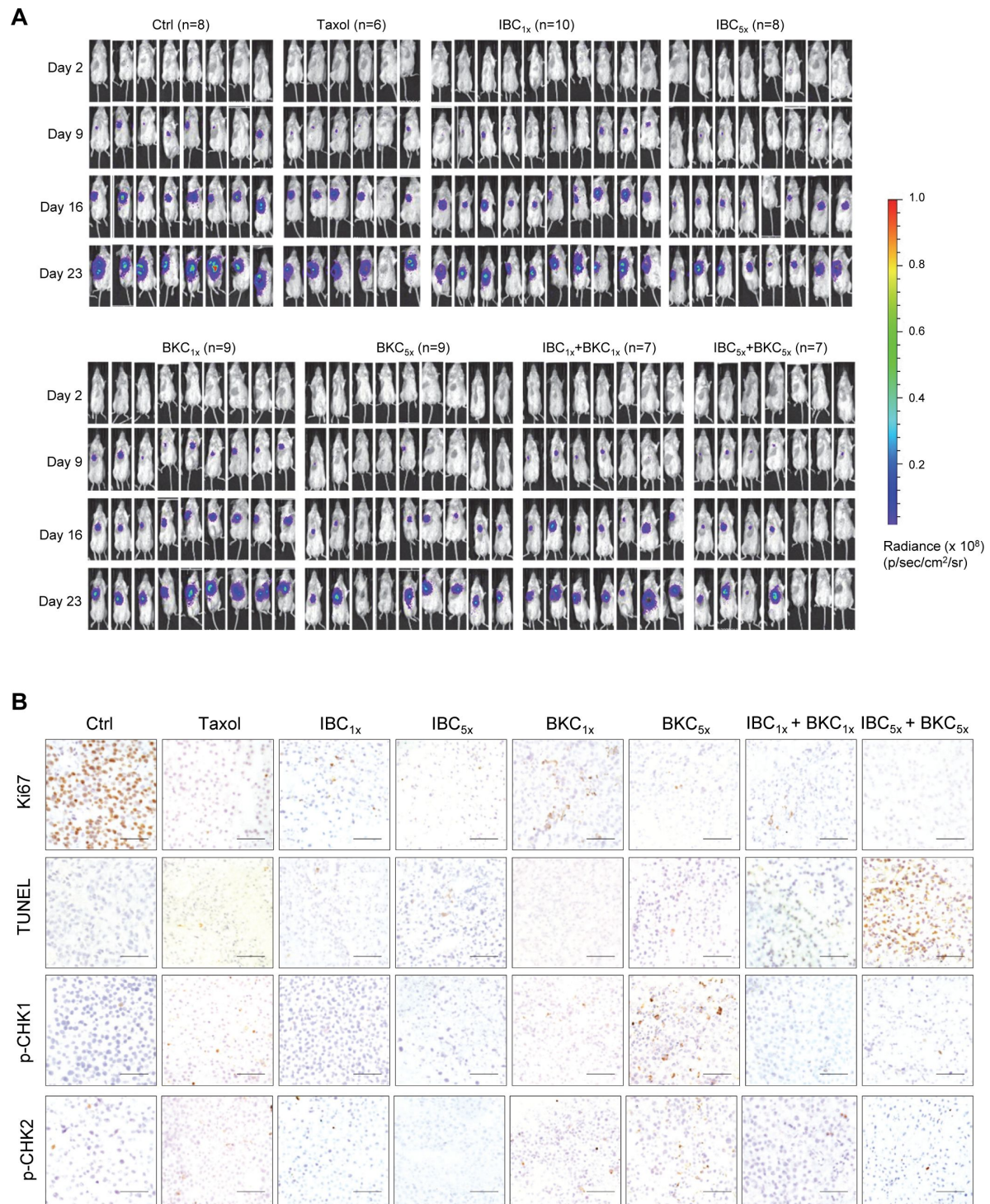


Fig. S6.

IBC and BKC extend survival and induce replication stress and DNA damage in a xenograft mouse model.

A. Luminometry images of MCF-7/Luc xenograft mice treated with PBS, Taxol, Isobavachalcone (IBC), Bakuchiol (BKC) or the combination IBC + BKC during treatments. **B.** Immunohistochemistry images of Ki67, TUNEL staining, pCHK1 (S345) and pCHK2 (Thr68) in xenografted tumor tissues.

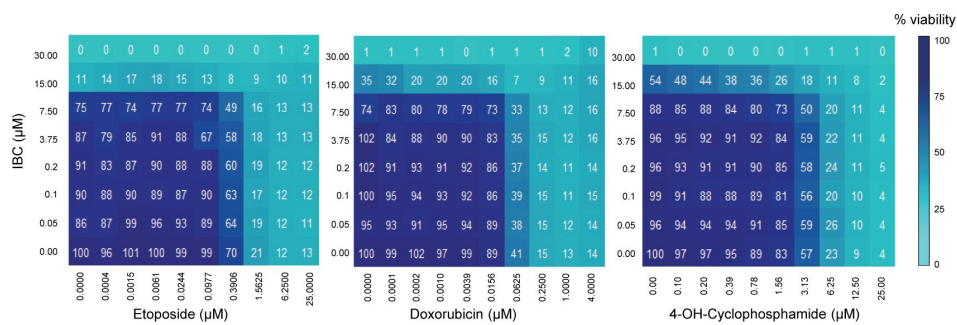


Fig. S7.

IBC potentiates the anti-cancer of chemotherapeutic agents in DLBCL.

U2932 cells were treated with indicated concentrations of etoposide, doxorubicin, or 4-OH-cyclophosphamide in combination with indicated concentrations of IBC for 72 hours. Cell viability was measured. Percentage of cell viability is indicated by the blue gradient in the matrix.

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Reviewer #1 (Public review):

The manuscript by Coquel et al. investigates the effects of BKC and IBC, two compounds found in *Psoralea corylifolia* in DNA replication and the response to DNA damage, and explores their potential use in cancer treatment. These compounds have been previously shown to affect different cellular pathways and the authors use transformed cancer cells of different origins and a non-transformed cell line to question if their combination is toxic in cancer versus non-cancer cells. They propose that BKC inhibits DNA polymerases while IBC targets CHK2. Their results show that both compounds do affect DNA replication, inducing replication stress and affecting double strand break repair. They also show that their combined use increases their toxicity in a synergistic manner.

Comments on current version:

The authors have addressed the main questions raised in the original manuscript. The new data provide stronger evidence supporting the inhibition of DNA polymerases by BKC and the effect of IBC on CHK2. In addition, the new data provides information about the potential mechanism of action of IBC in cells and xenograft models. Together, the revised manuscript has notably increased the relevance and impact of the results with stronger conclusions and better controlled experiments.

<https://doi.org/10.7554/eLife.104718.1.sa2>

Reviewer #2 (Public review):

Summary:

The manuscript: "Synergistic effect of inhibiting CHK2 and DNA replication on cancer cell growth" successfully demonstrates that the compounds BKC and IBC found in *Psoralea corylifolia* act synergistically to inhibit cancer cell proliferation, using a wide range of well-chosen methodologies. Moreover, the authors characterized the mechanisms of action of both drugs, which result in inhibition of cell proliferation. The use of multiple cell lines and the mice models makes the study robust and complete.

Significance:

The manuscript presents a well written study that offers new insights and contributions to the field. Although the inhibitors described have been known in science, the authors present convincingly their mode of action, which is either better characterized (for BKC) or inhibiting a different than previously suggested enzyme (for IBC). Authors also nicely pinpoint and explain the narrow window of concentrations when these two compounds act synergistically rather than additively. The analyses in multiple cell lines, mouse models and in combination with other cancer treatments, make this study of interest not only for fundamental researchers but also for translational scientists and industry.

Author response:

(1) General Statements

We thank all three reviewers for their constructive comments and suggestions. We also thank reviewers #2 and #3 for considering our work to be timely and of interest to the field, not only for basic researchers, but also for translational scientists and industry. We are now providing additional results to further support our hypothesis and hope that all reviewers will find that our manuscript is now ready for publication.

(2) Point-by-point description of the revisions

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

The manuscript by Coquel et al. investigates the effects of BKC and IBC, two compounds found in Psoralea corylifolia in DNA replication and the response to DNA damage, and explores their potential use in cancer treatment. These compounds have been previously shown to affect different cellular pathways and the authors use transformed cancer cells of different origins and a non-transformed cell line to question if their combination is toxic in cancer versus non-cancer cells. They propose that BKC inhibits DNA polymerases while IBC targets CHK2. Their results show that both compounds do affect DNA replication, inducing replication stress and affecting double strand break repair. They also show that their combined use increases their toxicity in a synergistic manner.

However, there are some major conclusions that are still not very well supported by the data: first, the differential effect on cancer and non-transformed cells; second, the direct link of BKC to the inhibition of DNA polymerases; and third, it is unclear if CHK2 is the relevant target for IBC in this context.

Regarding these points the authors should address the following issues:

(1) Most of the experiments use BJ fibroblasts as a control cell line. In order to evaluate if these compounds are preferentially toxic for cancer cells, the use of more than one non-transformed cell line is necessary. In addition, BJ cells are fibroblasts while most of the cancer cell lines employed are of epithelial origin. The authors could use MCF10 and RPE cells (both of epithelial origin) as control cell lines to complement the results and better support this claim.

We have now monitored the effect of IBC and BKC on the proliferation of MCF-7, MCF-10A and RPE-1 cells using the WST-1 assay and obtained similar results as for BJ and MCF-7 cells. These results are now included in the revised manuscript as Fig. S1A and S1B.

(2) In order to explore what are the targets of BKC and IBC Cellular Thermal Shift Assays (CETSA) could be used. Either by doing an unbiased mass spectrometry analysis of proteins stabilized by these compounds or by a direct analysis of candidate proteins by western blot (a similar approach has been used for IBC to show that it inhibits SIRT2 in Ren et al., 2024 Phytotherapy Res).

We thank this Reviewer for suggesting the use of the CETSA assay. We have now performed CETSA on MCF-7 cells and found that IBC stabilizes CHK2 but not CHK1, to the same extent as the commercial CHK2 inhibitor BML-277 used here as a positive control. These results are now shown in new Fig. 4G and 4H.

(3) For BKC in vitro polymerase assays could be carried out to show the direct inhibition of the DNA polymerase delta, for instance.

We have used high-speed *Xenopus* egg extracts to replicate ssDNA *in vitro* (Fig. S2C). This assay differs from the *in vitro* replication assay using low-speed *Xenopus* egg extracts (Fig. 2H) in that it only monitors elongation by replicative DNA polymerases (Pol δ and ϵ) and not earlier steps such as origin licensing and activation. The combined use of both low-speed and high-speed extracts strongly supports the view that BKC inhibits replicative DNA polymerases.

To confirm this result, we have also used CETSA to monitor BKC binding to different subunits of DNA Pol δ and Pol ϵ in MCF-7 cells and in *Xenopus* egg extracts (Fig. 3C-D Fig. S3). We found that BKC binds POLD1 and POLE, the catalytic subunits of Pol δ and ϵ respectively, but not the accessory subunit POLD3 nor PCNA. Together with our docking results and DNA fiber experiments, these data strongly support the view that BKC is a potent inhibitor of DNA Pol δ and Pol ϵ .

(4) In addition, the authors could analyze the integrity of replication forks by PCNA immunofluorescence analysis. The colocalization of PCNA and POLD or POLE subunits could also support the role of DNA polymerases as targets of BKC.

Our molecular docking results also show that BKC occupies the catalytic sites of DNA Pol δ and ϵ , which may not affect their subcellular localization and/or PCNA binding. Since our DNA replication assays, CETSA and DNA fiber analyses strongly support the view that BKC inhibits replicative DNA polymerases, we have not performed this additional experiment.

(5) In the case of IBC and the inhibition of CHK2, the authors should check the effect of IBC on the phosphorylation of BRCA1 on S988. The changes in CHK2 phosphorylation in Figure 3B are not convincing. The experiment should be repeated and the average of at least three experiments needs to be quantified.

We now provide evidence that IBC inhibits BRCA1 phosphorylation on S988. Western blots and quantification for three biological replicates are shown in Fig. 4C and Fig. S4H. Densitometric quantification of CHK2 phosphorylation on S516 from 3 biological replicates, along with statistical analysis, is now shown in Fig. S4G.

(6) To prove that CHK2 is the relevant target for IBC the authors could test if ATM and CHK2 knockout cells are more resistant to this compound, since it would prevent the phosphorylation of CHK2.

We have performed siRNA transfection targeting CHK2. The transfected cells died after 72 hours in culture, so we have been unable to determine whether CHK2-KD cells have increased resistance to IBC.

In addition to these experiments, I would suggest some other major improvements in the manuscript:

(1) The concentration of both compounds should be provided in molar units throughout the paper.

Thanks for pointing this out, we now use molar units throughout the paper.

(2) The authors do not clearly indicate the concentration that is employed in the different experiments, making it difficult to assess the results. For instance, Figure 2 does not

include the concentration in the legend or in the text. Time and concentration need to be clearly shown for each experiment.

The experimental conditions and inhibitor concentrations are now clearly indicated for each experiment.

(3) Some experiments are only repeated once (fiber assays) or twice (cell cycle analysis by flow cytometry). These experiments need to be repeated 3 times and the proper statistical analysis performed (comparison of the medians).

Superplots with biological replicates for all DNA fiber assays are now displayed. The number of biological replicates is now indicated in the legends and appropriate statistical analyses are used.

Other minor points or suggestions:

(1) Analyzing fork asymmetry would further support the direct effect of BKC on DNA polymerases.

The effect of BKC on fork asymmetry is now shown in Fig. 2F.

(2) A dose dependent analysis of BKC on the speed of DNA replication would also support this point.

Superplots of DNA fiber assays showing the effect of different concentrations of BKC on fork speed from three biological replicates are now included in Fig. 2E.

(3) Page 7: BKC reduces fork speed ...two-fold. This sentence is not very clear, it would be better to say that speed is half of the control.

This sentence was changed to “BKC reduced fork speed by a factor of two relative to untreated cells”.

(4) Figure 4G and S4D show contradictory results regarding the induction of Rad51 foci by IBC treatment. This needs to be clarified.

Figure 4G and S4D (now Fig. 5G and S5D) do not show contradictory results. In both cases, IBC treatment impaired the induction of RAD51 foci by IR or bleomycin.

(5) Page 12, Figure S5C is called for but it does not exist (probably meaning Figure S5B).

We apologize for this error, which has now been corrected.

Reviewer #1 (Significance):

The work by Coquel et al. aims at elucidating the use of BKC and IBC as a combined therapy to induce cell death in cancer cells by targeting DNA replication and CHK2. Both BKC and IBC have been previously shown to affect the proliferation of cancer cells. BKC has been shown to induce S phase arrest in an ATR dependent manner in MCF7 cells (Li et al., 2016 Front Pharm), while IBC induces cell death in MDA-MB-231 cells (Wu et al., 2022 Molecules). In this regard, the more interesting contribution of the manuscript is the potential identification of the targets of these compounds in cancer cells. The inhibition of CHK2 by IBC is quite compelling although it needs to be further proven. In contrast, the hypothesis that BKC inhibits DNA polymerases remains highly speculative. The results offer a limited advance in the knowledge of the mechanism of action of these two

compounds. Focusing on the action of IBC on CHK2 would increase the impact of the results. In this sense a very recent report has been published showing that IBC inhibits SIRT2 (Ren et al., 2024 Phyto Res), showing that IBC can affect multiple enzymes and processes. This should be taken into account for a further analysis of its mechanism of action.

In addition to the identification of the targets of BKC and IBC, the authors also focus on their combination for cancer treatment. This is based on the idea that blocking the DSB repair and inducing replication stress at the same time is an efficient approach to induce cancer cell death. This is not a new concept, since the loss of ATM sensitizes cancer cells to the inhibition of the replication stress response and several combination therapies have been put forward with the idea of generating replication stress and preventing the subsequent repair of the double strand breaks induced in these cells. Thus, the novelty here is limited, especially considering that the effect of BKC on DNA replication has already been described. Further, since its mechanism of action is unclear, it is difficult to ascribe the observed synergy to the speculated hypothesis. A deeper analysis of IBC as a CHK2 inhibitor would be more interesting, and the potential combination with other chemotherapy agents such as replication stress inhibitors, HU or DNA damaging agents. Also, the lack of a good control of non-transformed cells also reduces the relevance of the work.

In its current state, the interest of the manuscript is limited. The mechanistical advance is not strong enough and is not completely supported by the data, and the use of these compounds as a combination therapy does not provide new insights in cancer treatment. In my opinion, focusing on the inhibition of CHK2 by IBC and its potential use would broaden the impact of the results beyond the mere analysis of the action of these compounds.

We thank this reviewer for his/her constructive and insightful comments. We have followed his/her advice and focused our analysis on the action of IBC on CHK2. Using CETSA, we confirmed that IBC binds CHK2 to the same extent as BML-277 inhibitor, but does not bind CHK1. We also show that IBC inhibits BRCA1 phosphorylation on S988 and CHK2 phosphorylation on S516. Together with the results presented in the initial version of the manuscript, these data support the view that CHK2 is a key IBC target. We have also applied CETSA to DNA polymerases and confirmed that BKC directly targets DNA Pol δ and ϵ . Although it is unlikely that IBC and BKC will ever be used in combination therapies, the synergistic effect that we measured on cancer cells *in vivo* and *in vitro* indicates that IBC sensitizes cancer cells to endogenous replication stress and to exogenous sources of DNA damage, which could be used to replace BKC in combination therapies. For instance, our data indicate that IBC can be used in combination with drugs such as etoposide, doxorubicin or cyclophosphamide to potentiate their effect on drug-resistant lymphoma cell lines (DLBCL). As requested by this Reviewer, we have modified the discussion section to put more emphasis on IBC and CHK2 inhibitors and we hope that he/she will now find this revised version suitable for publication.

Reviewer #2 (Evidence, reproducibility and clarity):

In the manuscript by Coquel et al., the authors report their findings on the effect of 2 natural compounds from *Psoralea corylifolia* plant extracts on cancer cells. They show that these compounds, bakuchiol (BKC) and isobavachalcone (IBC), inhibit proliferation of cancer cells and tumor development in xenografted mice, particularly when used in combination. They further show that BKC inhibited DNA polymerases and induced replication stress, and show evidence that IBC inhibits Chk2 kinase activity and downstream double-strand break repair. Based on their findings, the authors conclude

that Chk2 inhibition and DNA replication inhibition represent a potential synergistic strategy to selecting target cancer cells.

Major:

(1) The data showing IBC is a Chk2 inhibitor is weak and more rigorous investigation is needed to establish this compound as a Chk2 inhibitor.

As indicate in our response to Reviewer #1, we have now analyzed the binding of IBC to CHK2 using the Cellular Thermal Shift Assay (CETSA) in MCF-7 cells. Our data clearly show that IBC binds to CHK2 but not CHK1. These results are now shown in Fig. 4G and 4H.

For one, the authors mention they screened 43 cell cycle-related kinases in vitro, but only show data for 8 kinases in their kinase activity screens. Of these 8 kinases, Chk2 is the most strongly inhibited, but there are no data shown for the other 35 kinases.

Data for all the protein kinases tested in the *in vitro* assay are now presented in Fig. S4D and S4E.

Additionally, the purpose of the CHK2 mutants should be discussed in the text.

The CHK2(I157T) mutation is linked to an increased risk of breast and colorectal cancers. CHK2(R145W) is associated with Li-Fraumeni Syndrome. Both mutations do not affect the basal kinase activity of CHK2. This information is now indicated in the legend of Fig. S4D.

Secondly, the western blot in Fig 3B, appears to show a very modest effect of IBC on Chk2 autophosphorylation and not that different from the effect of IBC on Akt phosphorylation in Fig S3a. Yet, the authors claim that IBC inhibits Chk2 but not Akt. To strengthen these blots, a known Chk2 inhibitor, such as the one shown in Fig 4 (BML-277) should be included as a positive control for pChk2 similarly to what was shown for Akt with MK-2206.

We have now replaced the western blot in Fig. 3B (now Fig. 4B) with another biological replicate. Quantifications and statistical analyses of biological replicates are shown in Fig. S4G. Overall, we observed a 50% reduction of CHK2 auto-phosphorylation in MCF7 cells treated with IBC, and a 20% reduction in AKT phosphorylation (Fig. S4A). There was no additional reduction in AKT phosphorylation when cells were treated with IBC in combination with MK-2206, compared to cells treated with MK-2206 alone. We now include the CHK2 inhibitor BML-277 as a positive control alongside with IBC to monitor CHK2 and CHK1 auto-phosphorylation in Fig. 4B, S4G, 4D and S4I, respectively.

Western blots showing a loss of phosphorylation of additional Chk2 targets is also needed. The manuscript mentions Brca1 S988 as a Chk2 substrate important for DSB repair. Showing the effect of IBC on this phosphorylation site would strengthen the conclusions.

We now provide evidence that IBC inhibits BRCA1 phosphorylation at S988. Western blots and quantification for three biological replicates are shown in Fig. 4C and S4H.

(2) The authors claim that the combination of IBC and BKC inhibit cell growth in a synergistic manner and that the "effect is more pronounce on cancer cells than on non-cancer cells." However, only 1 non-malignant cell line was used, and it was a fibroblast line. To make this claim, the authors need to show the effect in additional non-malignant cells, preferably with epithelial cell types.

We have now monitored cell proliferation using the WST-1 assay in two additional non-malignant cell lines, namely MCF-10A and RPE-1 cells. Cells were treated with IBC/BKC and their growth was compared to that of MCF-7 cells. These experiments yielded similar results to those obtained with BJ fibroblasts. These new data are now included in the revised version as Fig. S1A and S1B.

Minor:

(1) Densitometry data for all western blots should be shown with mean $\pm$ stdev of independent western blots.

Densitometry data for all western blots with biological replicates are now shown in supplementary figures.

(2) In Figure 1B the statistical test used to analyze cell number was not stated.

The statistical test is now indicated in Fig. 1B.

(3) In Figure 2A, the DAPI image for BKC is the merged image and should be replaced with just DAPI.

This error has now been corrected.

(4) In Figure 2B, the y-axis label says "yH2AX foci (MFI)". MFI and foci are not the same thing, and for yH2AX, the signal is often not focal. MFI of yH2AX is an appropriate measurement for replication stress, it's just not appropriate to equate MFI to foci.

We apologize for this labeling error, which has now been corrected.

(5) For the 53BP1 MFI and Rad51 MFI shown in Fig 4 and Fig S4, it is more appropriate to show the number of foci/cell as these are better indicators of breaks and repair sites. MFI is influenced by expression levels of the proteins and not necessarily the break/repair.

The numbers of 53BP1 and RAD51 foci are now shown.

(6) The data in Figures 5B and 5C are very difficult to read. Perhaps color-coat the lines/symbols.

We have now colored the graph to increase its readability.

Reviewer #2 (Significance):

The findings reported in this manuscript are timely, of interest to the field, and are mostly wellsupported by the experimental data. However, there are a few concerns that need to be addressed.

We are grateful to Reviewer #2 for his positive assessment of our manuscript. We hope that we have adequately addressed all of his/her specific concerns and that he/she will agree with the need to put more emphasis on IBC and CHK2 inhibition as requested by Reviewer #1.

Reviewer #3 (Evidence, reproducibility and clarity):

The manuscript: "Synergistic effect of inhibiting CHK2 and DNA replication on cancer cell growth" successfully demonstrates that the compounds BKC and IBC found in Psoralea

corylifolia act synergistically to inhibit cancer cell proliferation, using a wide range of well-chosen methodologies. Moreover, the authors characterized the mechanisms of action of both drugs, which result in inhibition of cell proliferation. The use of multiple cell lines and the mice models makes the study robust and complete. The manuscript presents a well written study that offers new insights and contributions to the field.

A few suggestions to improve the study:

(1) Given that both compounds BKC and IBC have already been previously described in the literature, it would be helpful for the reader to have them described better at the beginning of the study.

Thanks for pointing this out. We have now better described BKC and IBC at the beginning of the results section, as well as in the discussion. We agree that this could be helpful to readers.

(2) Addition of western blot quantifications over the number of experimental repeats is important specifically for Fig. 2C and Fig. 3C where partial effect of treatment on a signal level is reported.

The densitometry analysis of data shown in Fig. 2C and biological replicates are now shown in Fig. S2B. Quantification for Fig. 3C (now Fig. 4D) is shown in Fig. S4I.

(3) The quantification of mean intensity for 53BP1 and RAD51 foci should be exchanged with the quantification of number of foci per cell. While the quantification of γH2AX signal intensity is a correct representation of induction of this signal upon damage, foci formed by protein recruitment to DNA damage sites should be quantified by counting the number of foci, rather than signal in the whole cell/nucleus. These proteins exist before damage and are re-located in response to the damage.

Quantification of 53BP1 and RAD51 foci is now expressed as the number of foci per cell.

(4) Materials & Methods section is missing the methods for the experiment described in Fig. 1B. In summary, after addressing our few concerns, we believe the manuscript should be accepted for publication.

The WST-1 assay used for cell number quantification is included in “Reagents” in Material & Methods section.

Reviewer #3 (Significance):

The manuscript presents a well written study that offers new insights and contributions to the field. Although the inhibitors described have been known in science, the authors present convincingly their mode of action, which is either better characterized (for BKC) or inhibiting a different than previously suggested enzyme (for IBC). Authors also nicely pinpoint and explain the narrow window of concentrations when these two compounds act synergistically rather than additively. The analyses in multiple cell lines, mouse models and in combination with other cancer treatments, makes this study of interest not only for fundamental researchers but also for translational scientists and industry.

My field of expertise: DNA replication and replication stress across model systems.

We are grateful to Reviewer #3 for his/her very positive assessment of our work and we hope that he/she will find this revised version suitable for publication.

<https://doi.org/10.7554/eLife.104718.1.sa0>