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Benzo[a]pyrene-7,8-dihydrodiol promotes checkpoint activation and G2/M arrest in human bronchoalveolar carcinoma H358 cells

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Running title page

a) Running title: BPD activation of G2/M checkpoint

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are potent carcinogens that require metabolic activation inside cells. The proximate carcinogens PAH-diols can be converted to *o*-quinones by aldo-keto reductases (AKRs) or to diol-epoxides by cytochrome P450 (CYP) enzymes. We assessed the effect of BP-7,8-dihydrodiol (BPD) on proliferation in p53-null bronchoalveolar carcinoma H358 cells. BPD treatment led to a significant inhibition of proliferation and arrest in G2/M in H358 cells. The relative contribution of the AKR and CYP pathways to cell cycle arrest was assessed. Overexpression of AKR1A1 did not affect cell proliferation or cell cycle progression, and BP-7,8-dione (BPQ) did not cause any noticeable effect on cell growth, suggesting that AKR1A1 metabolic products were not involved in the anti-proliferative effect of BPD. On the other hand, blockade of CYP induction or inhibition of CYP activity greatly impaired the effect of BPD. Moreover, CYP induction by TCDD significantly enhanced the anti-proliferative effect of BPD. Mechanistic studies revealed that BPD caused a DNA damage response, Chk1 activation, and accumulation of phospho-Cdc2 (Y15) in H358 cells, effects that were impaired by an ATM/ATR inhibitor. Similar results were observed in human bronchoepithelial BEAS-2B cells, arguing for analogous mechanisms in tumorigenic and immortalized non-tumorigenic cells lacking functional p53. Our data suggest that a p53-independent pathway operates in lung epithelial cells in response to BPD that involves CYPs induction and subsequent activation of the ATR/ATM/Chk1 damage checkpoint pathway and cell cycle arrest in G2/M.

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Benzo[a]pyrene (BP), a five-ring polycyclic aromatic hydrocarbon (PAH) present in cigarette smoke, was among the first compounds recognized to exhibit carcinogenic activity in mice and implicated in the development of lung cancer in humans (Levin et al., 1977; Rubin, 2001). PAHs are pro-carcinogens that need to be metabolically activated to electrophiles in order to exert their deleterious effects. Three pathways have been proposed for their metabolic activation. The first pathway involves the formation of *anti*- and *syn*-diol epoxides catalyzed by members of the Cytochrome P450 family (CYP1A1 and CYP1B1) in conjunction with epoxide hydrolase (EH) (Penning et al., 1999). In the case of BP, the end product is *anti*-BDPE, a highly mutagenic and tumorigenic metabolite. Reaction of PAH-diol epoxides with DNA leads to stable adducts (Jennette et al., 1977), which may result in misreplication and mutagenesis. *anti*-BDPE has been shown to mutate the proto-oncogene *c-H-ras* via G to T transversions (Vousden et al., 1986) and to target hot spots in the *p53* tumor suppressor gene (Rodin and Rodin, 2005). A second pathway of metabolic activation involves the formation of radical cations by CYP peroxidase (Cavalieri and Rogan, 1995), which can react with DNA to yield unstable depurinating adducts (Stack et al., 1995). The formation of apurinic sites, if unrepaired, can lead to G to T transversions in target genes (Chakravarti et al., 1995). The third metabolic pathway involves aldo-keto reductases (AKRs) that oxidize PAH *trans*-dihydrodiols to generate reactive and redox-active *o*-quinones (Penning et al., 1999). The *o*-quinones can form stable and depurinating DNA adducts (Balu et al., 2004; McCoull et al., 1999; Shou et al., 1993). Autooxidation of the intermediate catechols and/or subsequent redox cycling of the *o*-quinones generate reactive oxygen species (ROS) capable of modifying DNA as well as other macromolecules (Penning et al., 1999).

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It is well established that oxidative and genotoxic stress activates checkpoint mechanisms to arrest cells with damaged DNA prior to their entry into phases of DNA replication and cell division. Such protective mechanisms are initiated by phosphoinositide-3 kinases (PI3K)-like kinases that sense DNA damage (DNA-PK, ATM and ATR), which in turn activate serine/threonine kinases Chk1 and Chk2 and lead ultimately to cell cycle arrest and/or apoptosis (Zhou and Elledge, 2000).

Limited information is available regarding the relative contribution of the various PAH metabolic pathways and PAH metabolites to cell proliferation and cell cycle progression. Studies have shown that BP and its metabolites could promote proliferation in mammary MCF-10A (Burdick et al., 2003) and lung squamous carcinoma 128-88T cells (Oguri et al., 2003), or inhibit cell growth in other cell types, such as in normal human bronchoepithelial NHBE cells (Fields et al., 2004). p53 mutations occur in more than half of lung tumors (Rodin and Rodin, 2005) and can be detected in the normal epithelium of smokers (Mao et al., 1997), an event that could eventually provide a proliferative growth advantage. Human colon carcinoma, breast carcinoma and lung cancer cells fail to arrest in G1 in response to treatment with PAHs (Dipple et al., 1999), a situation that could lead to replication of DNA on a damaged template. Studies using PAH *o*-quinones revealed that they could confer a proliferative advantage via different mechanisms, including the activation of the epidermal growth factor receptor (EGFR), Erk and Akt (Burdick et al., 2003), an indication of the complexity in the response pattern of PAH metabolites on mitogenicity.

In this study we examined the effect of the proximate carcinogen BP-7,8-diol (BPD) on the proliferation of p53-null bronchoalveolar carcinoma H358 cells and immortalized non-tumorigenic human bronchoepithelial BEAS-2B cells, as well as the relative contribution of the

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CYP- and AKR-dependent pathways. We found that exposure of these cells to BPD leads to growth arrest and the accumulation of cells in G2/M. Interestingly, metabolic activation of BPD to BPQ via the AKR pathway was dispensable for this effect, which instead depends on the metabolic activation to BPDE via CYPs. DNA damage induced by BPD via the checkpoint kinase Chk1 played an essential role in this growth inhibitory effect.

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MATERIALS AND METHODS

Chemical and reagents. Cell culture media and reagents were purchased from Invitrogen Co. (Carlsbad, CA). (±)-BP-7,8-diol, (±)-*anti*-BPDE, BP-7,8-dione and TCDD were obtained either from NCI Chemical Carcinogen Standard Reference Repository (Midwest Research Institute, Kansas City, MO) or from Dr. Ronald G. Harvey (The Ben May Institute for Cancer Research, University of Chicago). All BP metabolites were checked for purity and validated by LC/MS. TMS was obtained from Tocris Cookson Ltd. (Ellisville MO). DFB and caffeine were purchased from Sigma-Aldrich (St. Louis, MO). All PAHs are potentially hazardous and have been handled in accordance with NIH Guidelines for the Use of Chemical Carcinogens (NIH Pub. No. 81-2385).

Cell culture. Human bronchoepithelial carcinoma H358 cells were obtained from ATCC and cultured at 37^{o}C in a humidified 5% CO_2 atmosphere in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. H358 cells stably overexpressing AKR1A1 (Jiang et al., 2005) were grown in the same medium supplemented with G418 (0.4 mg/ml). Human bronchoepithelial immortalized BEAS-2B cells were obtained from ATCC and cultured in plates coated with fibronectin (Roche), albumin (Roche), and collagen (Inamed, Freemont, CA), using LHC-9 medium.

Cell proliferation and cell cycle analysis. Cells (2 x 10^5) per well were seeded in triplicate in 12-well plates and 24 h later treated for 6 h with different concentrations of BPD, BPQ or BPDE in HBSS. After extensive washing, complete RPMI medium was added and, at different

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intervals, cells were trypsinized and counted in a hemocytometer. For the MTS assay, 1 x 10^4 cells/well were seeded in triplicates in 96-well plates. Cells were treated with the various PAHs and OD_{490} was determined directly after adding the CellTiter96 Aqueous One Solution Reagent (Promega, Madison, WI). For determination of cell cycle profiles, 6 x 10^5 cells seeded in 60 mm dishes were subject to various treatments, stained with propidium iodide (0.1 mg/ml), and analyzed by flow cytometry, as previously described (Nakagawa et al., 2005).

Western Blot analysis. Cells were lysed in a buffer containing 50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, and 5% β-mercaptoethanol. Extracts (40 μg protein/lane) were subject to SDS-PAGE and transferred to nitrocellulose membranes (Millipore, Bedford, MD). After blocking with 5% milk in 0.05% Tween-20/PBS, membranes were incubated with the primary antibody as directed by manufacturer. Anti-mouse or anti-rabbit horseradish peroxidase (BioRad) were used as secondary antibodies. Bands were visualized with the enhanced chemiluminescence (ECL) Western blotting detection system. The following antibodies were used (1:1000 dilution): anti-AKR1A1 polyclonal antiserum (provided by Dr. John Hayes, University of Dundee), anti-Chk1, anti-phospho-Chk1 (Ser345), anti-phospho-Cdc2 (Tyr15), anti-Cdc2 (Cell Signaling, Danvers, MA), anti-cyclin A, anti-cyclin B1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-H2A.X (Ser139) (AbCam, Cambridge, MA), and anti-actin (Sigma).

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RESULTS

BPD induces G2/M-arrest of H358 bronchoalveolar carcinoma cells

In the first set of experiments we investigated whether BPD affects the proliferation of H358 bronchoalveolar carcinoma cells. Asynchronous H358 cells were treated with increasing concentrations of BPD (30 nM-1 μM) for 6 h. After removal of the compound by extensive washing, cell number was determined at regular intervals (24-72 h). As shown in Fig. 1A, BPD treatment caused a dose-dependent reduction in proliferation. Using the MTS assay (Fig. 1B) we observed a similar inhibition of proliferation by BPD. No significant cell death was observed up to 1 μM BPD, as judged by quantification of cells with nuclear fragmentation after DAPI staining (Fig. 1B) or by the analysis of the sub-G0/G1 population using flow cytometry (Fig. 1C).

Analysis of cell cycle distribution revealed a dose-dependent elevation of cells in G2/M upon BPD treatment. While control ansynchronous H358 cultures showed ~ 25% of cells in G2/M, this percentage doubled in response to 300 nM BPD (Fig. 1C). A slight increase in S phase cells could also be detected. Analysis of relevant cell cycle markers showed significant elevations in cyclin A and cyclin B1 levels in BPD-treated cells. High phospho-Cdc2 (Y15) levels were also observed in BPD-treated cells, arguing for an impaired entry in mitosis (Fig. 1D).

G2 arrest in response to BPD is independent of its metabolic transformation to BPQ

The proximate carcinogens PAH-diols can be metabolized either to PAH-diol epoxides by CYPs or to PAH-*o*-quinones by AKR isozymes. PAH-*o*-quinones are highly redox active

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molecules with complete carcinogenic potential, and they have been shown to produce cytotoxicity by alkylation or oxidative stress (Penning et al., 1999). Furthermore, these compounds affect mitogenic pathways, such as those involving EGFR and PKC activation (Burdick et al., 2003; Yu et al., 2002). To determine whether the BPD conversion to BPQ plays a role in growth arrest we used two individual clones of H358 cells overexpressing AKR1A1 (Fig. 2A). These stable cell lines show increased AKR enzymatic activity with respect to parental cells (Jiang et al., 2005). A similar growth inhibitory response to BPD was observed both in parental and AKR1A1 overexpressors (Fig. 2B), and their cell cycle profiles were essentially identical (data not shown). Direct treatment of H358 cells with BPQ did not cause any significant reduction in cell viability (data not shown) or alterations in cell cycle distribution (Fig. 2C). These results suggest that the anti-proliferative effect of BPD was independent of its metabolism by AKRs.

BPD induces arrest in G2/M via the CYP pathway

CYP1A1 and CYP1B1 are key enzymes involved in PAH activation in lung (Jiang et al., 2005; Nebert et al., 2000). These enzymes are inducible via transactivation by AhR ligands such as planar aromatic compounds, 3-methyl-cholanthrene, β-naphthoflavone, and various PAHs (Nebert et al., 2000). TCDD strongly induced CYP1A1 and CYP1B1 mRNA levels (data not shown), which fits with the previously observed 70-fold increase in CYP activity in TCDD-treated H358 cells (Jiang et al., 2006). Interestingly, TCDD sensitized H358 cells to the anti-proliferative effect of BPD (Fig. 3A), as well as to the accumulation of cells in G2/M (Fig. 3B). Once induced, CYPs produce BPDE (Jiang et al., 2006), suggesting that this metabolite could be responsible for the anti-proliferative effect. Indeed, treatment of H358 cells with BPDE caused a

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significant reduction in proliferation and accumulation of cells in G2/M and S phases (Fig. 3C and 3D).

To further demonstrate the relevance of the conversion to BPDE in the growth inhibitory effect of BPD we used the AhR antagonist DFB, and the CYP1 selective inhibitor TMS (Jiang et al., 2006). As shown in Fig. 4A, the anti-proliferative effect of BPD was impaired by pretreatment with either DFB or TMS. Moreover, in H358 cells treated with TMS, BPD-induced accumulation of cells in G2/M was markedly reduced (Fig. 4B). Thus, conversion to BPDE by CYPs is an essential step for the growth arrest caused by BPD.

BPD activates a DNA damage checkpoint in H358 lung cancer cells

It is well established that BPDE forms stable-N2-dGuo adducts with DNA (Buterin et al., 2000; Ruan et al., 2006). While double stranded breaks (DSBs) are not directly generated by BPDE, they may be produced as byproducts of the repair mechanism, an effect also observed in lung cancer cells (Guo et al., 2002; Zhou et al., 2006). p53-null cells arrest either in S or G2 checkpoints to repair damaged DNA and preserve their genomic stability (Zhou and Elledge, 2000). Thus, it is likely that in p53-null H358 cells BPD-induced arrest in G2 involves checkpoint activation. BPD treatment of H358 cells led to BPDE-DNA adduct formation (Ruan et al., 2006) as well as elevated levels of histone 2A.X phosphorylation (Fig. 5A), a marker of DNA damage (DSBs) (Zhou et al., 2006). DSBs lead to p53-independent G2 arrest through the activation of ATM and ATR kinases, and their downstream kinases Chk1 and Chk2 (Khanna and Jackson, 2001; Zhou and Elledge, 2000). Western blot analysis revealed a significant elevation in phospho-Chk1 (S345) levels in H358 cells treated either with BPD (Fig. 5A) or BPDE (Fig. 5B). On the other hand, the AKR metabolite BPQ failed to activate Chk1 in H358 cells (Fig. 5B).

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Caffeine, an inhibitor of ATM and ATR (Sarkaria et al., 1999), prevented Chk1 activation in response to BPD (Fig. 5C). Moreover, caffeine rescued the anti-proliferative effect of BPD (Fig. 5D) as well as the accumulation of cells in G2/M (Fig. 5E). Next, we evaluated the effect of pharmacological modulators of the AhR/CYP pathway on the checkpoint response. Figure 5F shows that TCDD markedly enhanced BPD-induced phosphorylation of Chk1. On the other hand, either the CYP inhibitor TMS or the AhR antagonist DFB blocked the elevation in phospho-Chk1 (S345) levels by BPD.

BPD inhibits proliferation of immortalized bronchoepithelial cells via activation of Chk1 pathway

In the last set of experiments, we assessed the relevance of our findings in a model of immortalized bronchoepithelial cells (BEAS-2B). BEAS-2B were derived from noncancerous individuals, and were immortalized by infection with a SV-40/AD-12 hybrid virus (Reddel et al., 1988), which binds to the p53 protein and causes its loss of function. These cells are non tumorigenic in athymic mice. BPD treatment caused a dose-dependent reduction in BEAS-2B proliferation (Fig. 6A). Cell cycle studies revealed an accumulation of BPD-treated cells in S and G2/M (Fig. 6B). As previously shown in H358 cells, phospho-H2A.X and phospho-Chk1 levels were significantly elevated in response to BPD (Fig. 6C). Moreover, the ATM/ATR inhibitor caffeine blocked the inhibitory effect of BPD on cell proliferation and cell cycle progression in BEAS-2B cells (Fig. 6B and 6D).

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DISCUSSION

PAHs comprise a large group of environmental pollutants that in many cases have been reported to possess tumor initiating activity or even act as complete carcinogens. While these compounds have been extensively studied for their genotoxicity, epigenetic effects may also play a role in their carcinogenic activity, and indeed PAHs have been shown to promote proliferation in breast cancer cells, osteosarcoma cells, and osteoblasts (Burdick et al., 2003; Pliskova et al., 2005; Tsai et al., 2004). Studies using pharmacological inhibitors suggest that this stimulatory effect of PAH is mediated by MAPKs and PI3K (Burdick et al., 2003; Tsai et al., 2004). However, a growth inhibitory response to PAHs has been reported in other cell types.

In this study we investigated the effects of the proximate carcinogen BPD and its metabolites on proliferation and cell cycle regulation in p53-null H358 bronchoalveolar carcinoma cells. A main goal was to determine whether the effects of BPD on cell proliferation were mediated by products of CYP metabolism and/or AKR metabolism. Cancer cells exposed to BPDE fail to undergo a p53-mediated arrest in G1 (Khan and Anderson, 2001; Khan et al., 1999), and in turn they delay progression through S phase (Guo et al., 2002). It has been proposed that such evasion of G1 arrest may contribute to the initiation and progression of tumors. In contrast, in primary and MCF-10A human mammary epithelial cells, BP-*o*-quinones stimulate EGF signaling and proliferation via ROS generation (Burdick et al., 2003; Davis et al., 2001). Our studies in both p53-deficient H358 and BEAS-2B cells determined that BPD treatment causes a reduction in proliferation, due to arrest of cells in G2/M. This effect occurs in the nanomolar range. We provide strong evidence that the AKR and CYP metabolic pathways do not have equivalent roles in mediating the anti-proliferative effect of BPD. Surprisingly, the

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conversion of BPD to BPQ mediated by AKR1A1 does not account for the inhibitory effect of BPD on H358 cell proliferation. It has been previously reported that in AKR1A1 overexpressing cells, BPD is converted to BPQ, and BPQ can induce CYP1B1 (Jiang et al., 2006). Although one would expect that such induction could redirect BPD metabolism to BPDE formation, this is not observed at the concentrations and times that we have used in our experiments. On the other hand, pharmacological manipulation of the AhR pointed to an essential role for CYPs in the anti-proliferative effect of BPD. Moreover, direct treatment of H358 cells with BPDE caused similar reduced proliferation. A number of studies have established that BPDE forms adducts with DNA (Buterin et al., 2000), which correlates with lower rates of DNA synthesis and cell proliferation (Schwerdtle et al., 2002). BPDE-adducted DNA can elicit DSBs indirectly by single-stranded DNA nucleotide excision repair intermediates that are prone to breakage (Buterin et al., 2000; Stark and Taylor, 2006). Our study is the first in assessing the effects of the intracellular production of BPD metabolites on G2 checkpoint activation, and suggests that it is mediated by BPDE formation. Recent studies demonstrated that parental H358 cells treated with BPD for the times used in our experiments (6 h of incubation) form BDPE adducts [1]. Other evidence also supports the involvement of BPDE in the G2 checkpoint activation. First, we found a significant elevation in phospho-H2A.X levels in response to BPD and BPDE. Induction of this marker of DSBs in response to BP has been reported (Zhou et al., 2006), and could reflect the BPDE-mediated DNA damage. Once formed, DSBs may trigger the checkpoint signaling cascade (Bartek et al., 2004). Second, we found that Chk1 becomes phosphorylated in response to BPD and BPDE treatment. Third, pretreatment with caffeine, which inhibits ATM/ATR kinases (Sarkaria et al., 1999), blocked BPD-induced phosphorylation of Chk1, and in addition it rescued the anti-proliferative effect of BPD in H358 and BEAS-2B cells. Studies by Guo and colleagues

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in H1299 metastatic cells showed a reduction in DNA synthesis after BPDE treatment, which involves ATM and Chk1 activation (Guo et al., 2002). Taken together, these results reveal that BPD-induced inhibition of proliferation is mediated by the CYP pathway and triggered by the activation of checkpoint kinases in response to DNA damage.

Our studies also establish that the activation of the Chk1-dependent checkpoint leads to arrest of H358 cells in the G2/M phase of the cell cycle. The accumulation of H358 cells in G2/M correlates with elevated cyclin A and cyclin B levels. Western blot analysis also showed higher levels of phosphorylation of Tyr 15 in Cdc2, which may account for the p53-independent arrest. The Cdc2 is kept inactive by phosphorylation on Tyr15 and Thr14 by the kinases Wee1 and Myt1 respectively. At the onset of mitosis, Cdc2 is dephosphorylated by the Cdc25 phosphatases (Stark and Taylor, 2006). Recent evidence also points to mutually integrated roles of the checkpoint machinery in the activation of DNA repair, chromatin remodeling, modulation of transcriptional programs, and permanent cell-cycle withdrawal (cellular senescence) or cell death (Bartek et al., 2004; Zhou and Elledge, 2000). It would be interesting to pursue studies on the long-term effects of BPD treatment on survival of lung epithelial cells.

In summary, our studies identified a mechanism by which the proximate carcinogen BPD induces G2 arrest in p53-null lung cells, via the ATM/ATR/Chk1 pathway. In support of our studies, others have shown the activation of a Chk1-dependent G2 checkpoint in the absence of p53 in response to genotoxic stress, such as IR, UV, or oxidative stress (Koniaras et al., 2001; Macip et al., 2006). The fact that Chk1 activation in response to BPD or BPDE occurs in non-tumorigenic, immortalized human bronchoepithelial BEAS-2B cells, suggests a protective role for this pathway in response to carcinogens in lung epithelial cells.

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Footnotes

[1] Ruan, Q., Gelhaus, S.L., Penning, T.M., Harvey, R.G., and Blair, I.A. Alko-Keto reductase- and cytochrome P450-dependent formation of benzo[a]pyrene-derived DNA adducts in human bronchoalveolar cells. Submitted for publication.

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FIGURE LEGENDS

Fig. 1. BPD inhibits G2/M progression of bronchoalveolar carcinoma H358 cells. H358 cells were treated with different concentrations of BPD (30nM-1μM) or vehicle (Control) for 6 h. A, cell number was determined at regular intervals. B, MTS assay (top) and apoptotic cells (bottom) in H358 cells 48 h after treatment. For the MTS assay, results are expressed as % of reduction of proliferation respect to control. Apoptosis was determined after DAPI staining counting 300 cells. C, cell cycle distribution was determined by flow cytometry 48 h after treatment. Data is represented as mean ± S.D. of triplicate/duplicate samples (n=3). D, Western for relevant cell cycle markers on BPD-treated cells (300 nM for 6 h). For A, B and C, data is expressed as mean ± standar deviation (S.D.) of triplicate samples of a representative independent experiment. Two additional experiments gave similar results (n=3).

Fig. 2. BPQ production by AKR1A1 enzyme does not inhibit cell proliferation. The effect of BPD on AKR1A1 overexpressing (clones *A* and *B*) and parental (P) H358 cells was compared. A, levels of expression of AKR1A1 in parental and stably transfected clones of H358 cells determined by Western blot using a specific polyclonal antibody. B, cells were treated with different concentrations of BPD (30 nM-300 nM), and 48 h later cell number was determined. C, H358 cells were treated with different concentrations of BPQ (30 nM-1 μM) for 6 h, and 48 h later cell cycle profiles were analyzed by flow cytometry. In all cases data represent the mean ± S.D. of triplicates samples. Similar results were observed in 2-3 independent experiments.

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Fig. 3. TCDD treatment increases BPD anti-proliferative effect. A-B, H358 cells were treated with 10 nM TCDD (+TCDD), or medium alone (Control) for 6 h, followed by additional 6 h with BPD (30 nM-300 nM). A, cell number determined 48 h after treatment. Data is expressed as mean ± S.D. of triplicates. Three independent experiments gave similar results. B, cell cycle distribution 48 h after treatment. C and D, H358 cells were treated with different concentrations of BPDE for 6 h and 48 h later, cell number (C) and cell cycle profiles (D) were evaluated. Data is expressed as mean ± S.D. of triplicates from a representative experiment. Similar results were observed in 2-3 additional experiments.

Fig. 4. Inhibition of CYPs rescues the anti-proliferative effect of BPD. H358 cells were treated with either 1 μM difluorobenzuron for 6 h (+DFB), or TMS for 1 h (+TMS), and then with BPD (1 μM, 6 h). 48 h after treatment, cell number (A) and cell cycle distribution (B) were determined. Results are expressed as mean ± S.D of triplicate samples. Similar results were observed in 2 additional experiments.

Fig. 5. BPD induces activation of Chk1-checkpoint. A, H358 cells were treated either with medium alone (0 h) or BPD (1μM) for different times (1-12 h), and levels of phospho-Chk1 (S345) and phospho-H2A.X (S139) were determined by Western blot. B, similar experiments as in A were carried out with either BPDE or BPQ (3μM, 0-120 min). C-E, H358 cells were pre-treated with caffeine (5 mM, 1 h) and then treated with BPD (300 nM, 6 h). C, Western blot analysis 24 after treatment. D, cell number determined 48 h after treatment. E, cells cycle distributions 48 h after treatment. For D and E, data is expressed as mean ± S.D. of triplicates. Similar results were observed in 3 independent experiments. **, $p<0.01$.

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Fig. 6. BPD activates a Chk1 checkpoint in non-tumorigenic bronchoepithelial BEAS-2B cells. A, BEAS-2B cells were treated with different concentrations of BPD (30 nM-300 nM) for 6 h and cell number was determined at 48 h after treatment. C, representative Western blot 24 h after treatment. B and D, BEAS-2B cells were pretreated with caffeine (5 mM, 1 h) and then subject to BPD treatment (300 nM, 6 h). After 48 h cell cycle distribution (B) and cell number (D) were determined. Data in A, B and D are expressed as mean ± S.D. of triplicates. Similar results were obtained in 2-3 independent experiments. **, $p<0.01$.

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Figure 1

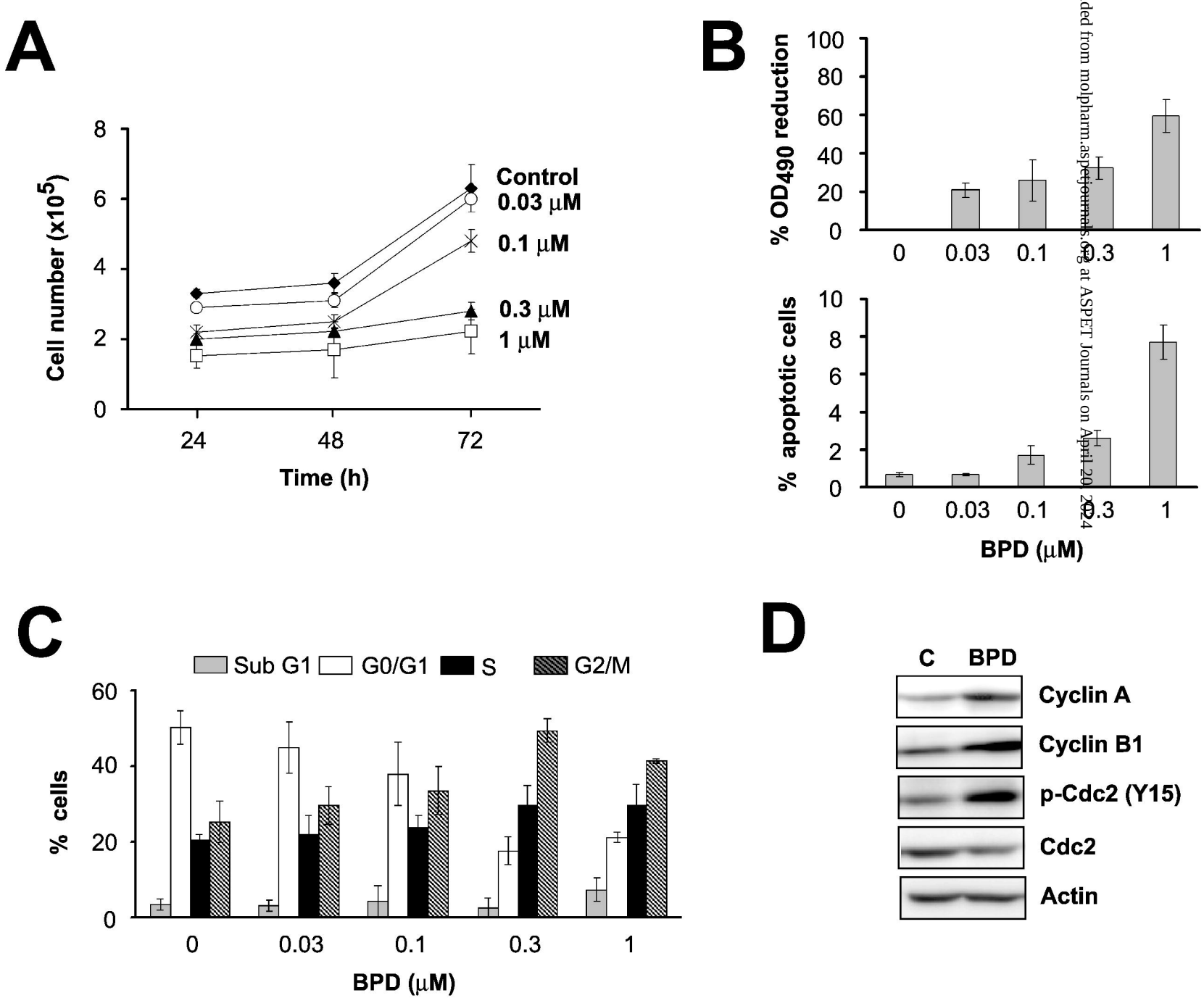

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Figure 2

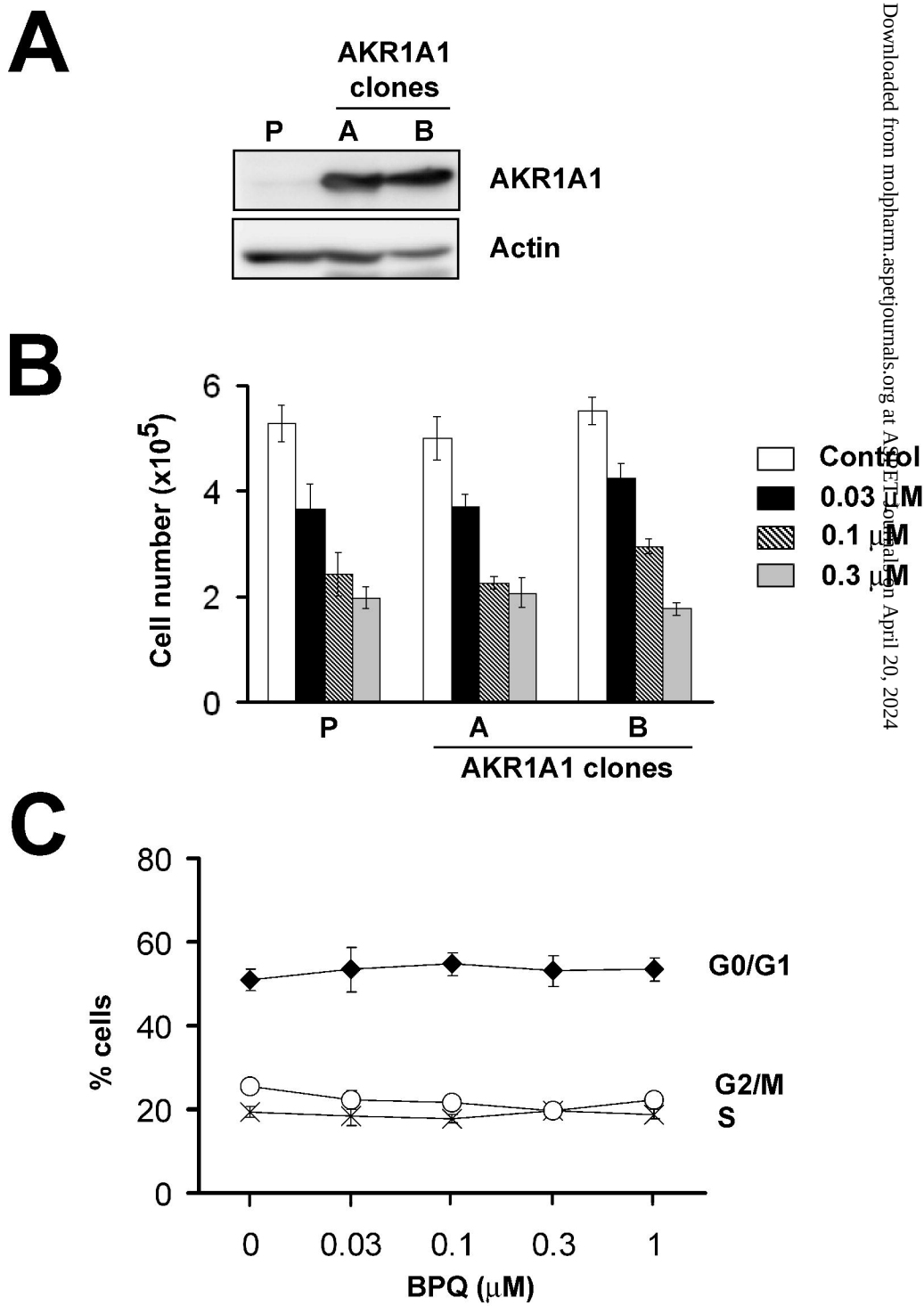

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Figure 3

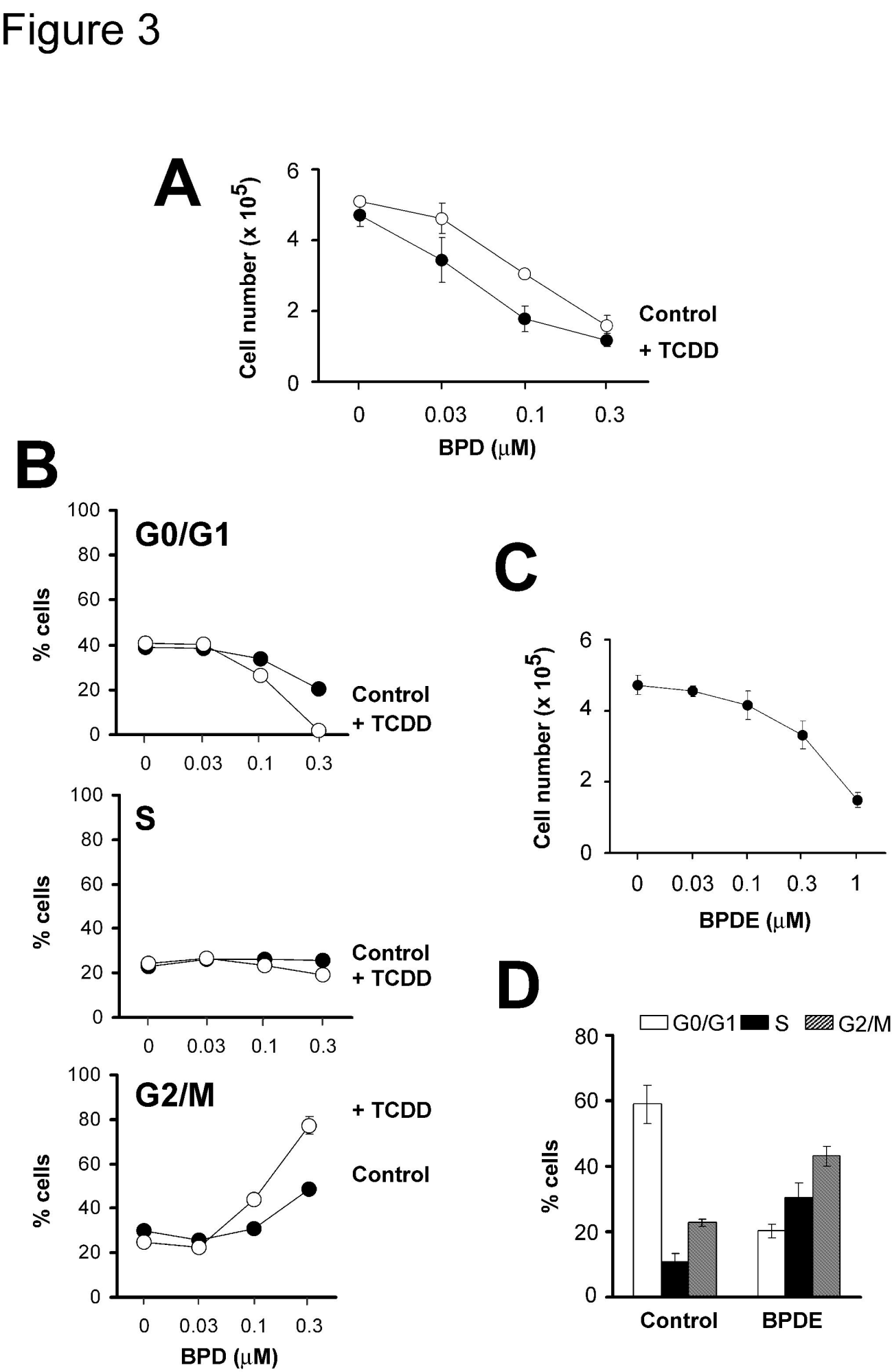
A
Cell number (x 10^5)
6
4
2
0
0
0.03
0.1
0.3
BPD (μM)
Control
+ TCDD
B
G0/G1
% cells
100
80
60
40
20
0
0
0.03
0.1
0.3
Control
+ TCDD
S
% cells
Control
+ TCDD
G2/M
% cells
+ TCDD
Control
BPD (μM)
C
Cell number (x 10^5)
6
4
2
0
0
0.03
0.1
0.3
1
BPDE (μM)
D
G0/G1
S
G2/M
% cells
80
60
40
20
0
Control
BPDE

Figure 4

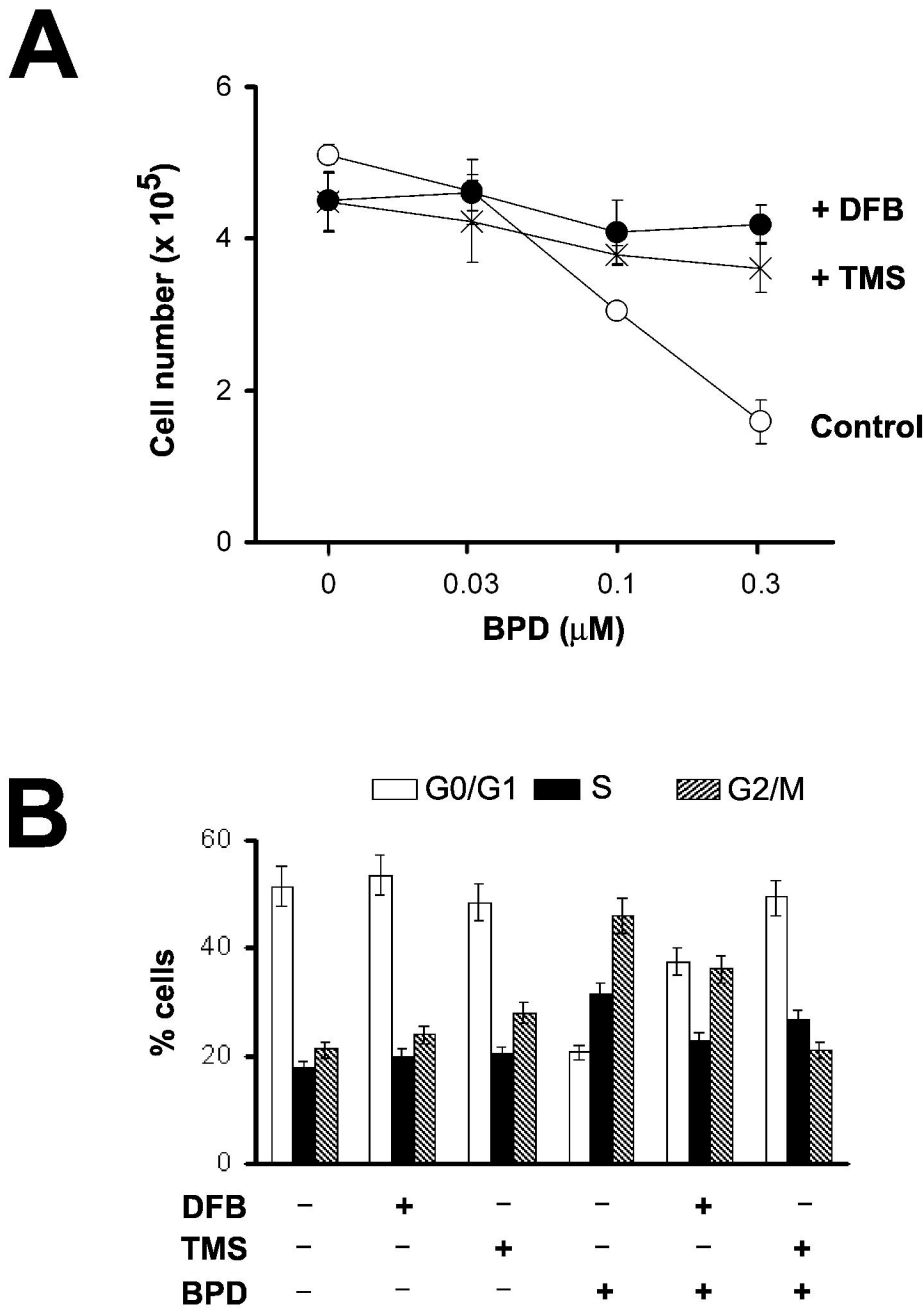
A
Cell number (x 10^5)
6
4
2
0
0
0.03
0.1
0.3
BPD (μM)
+ DFB
+ TMS
Control
B
G0/G1
S
G2/M
% cells
60
40
20
0
DFB – + – – + –
TMS – – + – – +
BPD – – – + + +

Figure 5

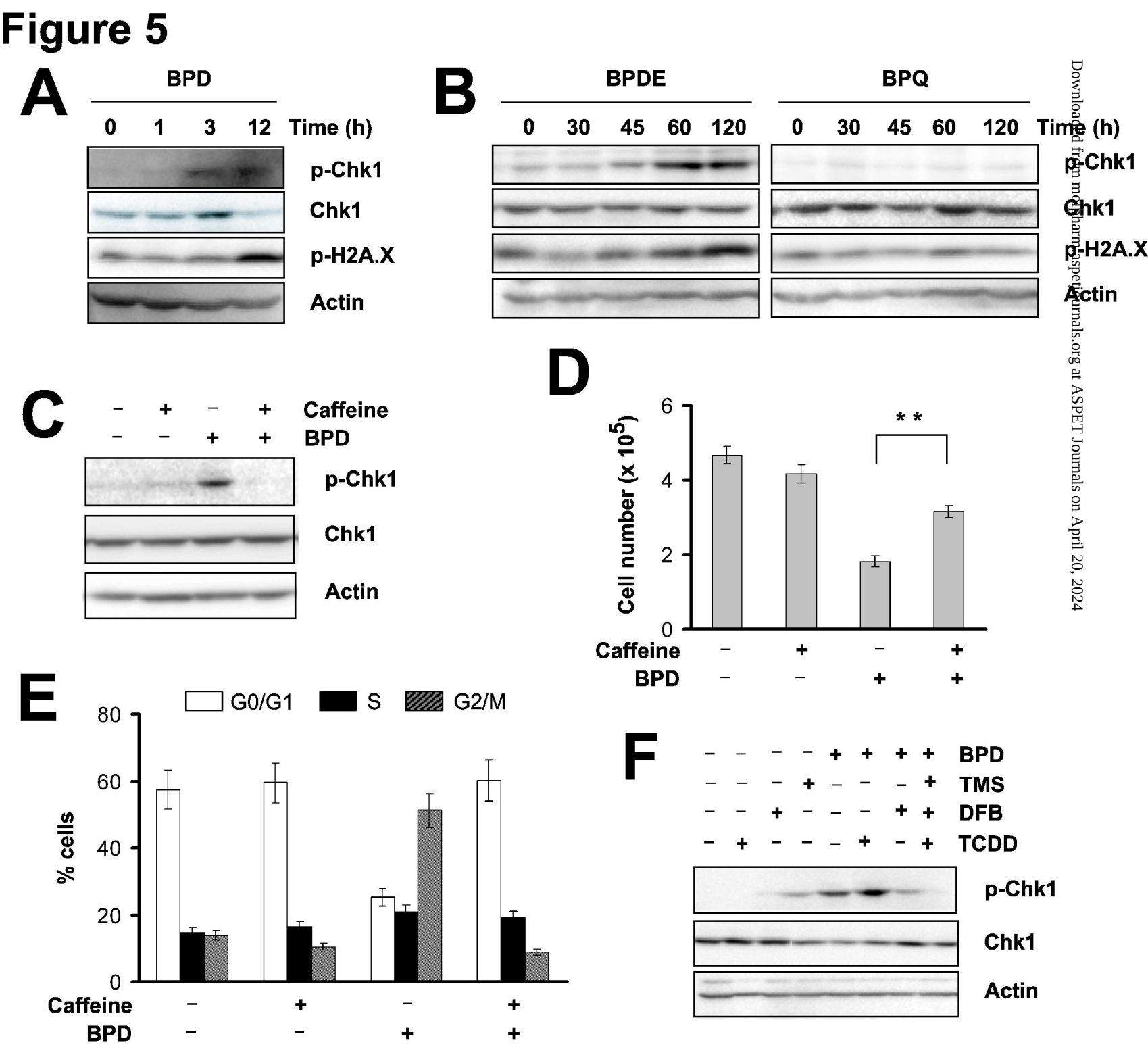

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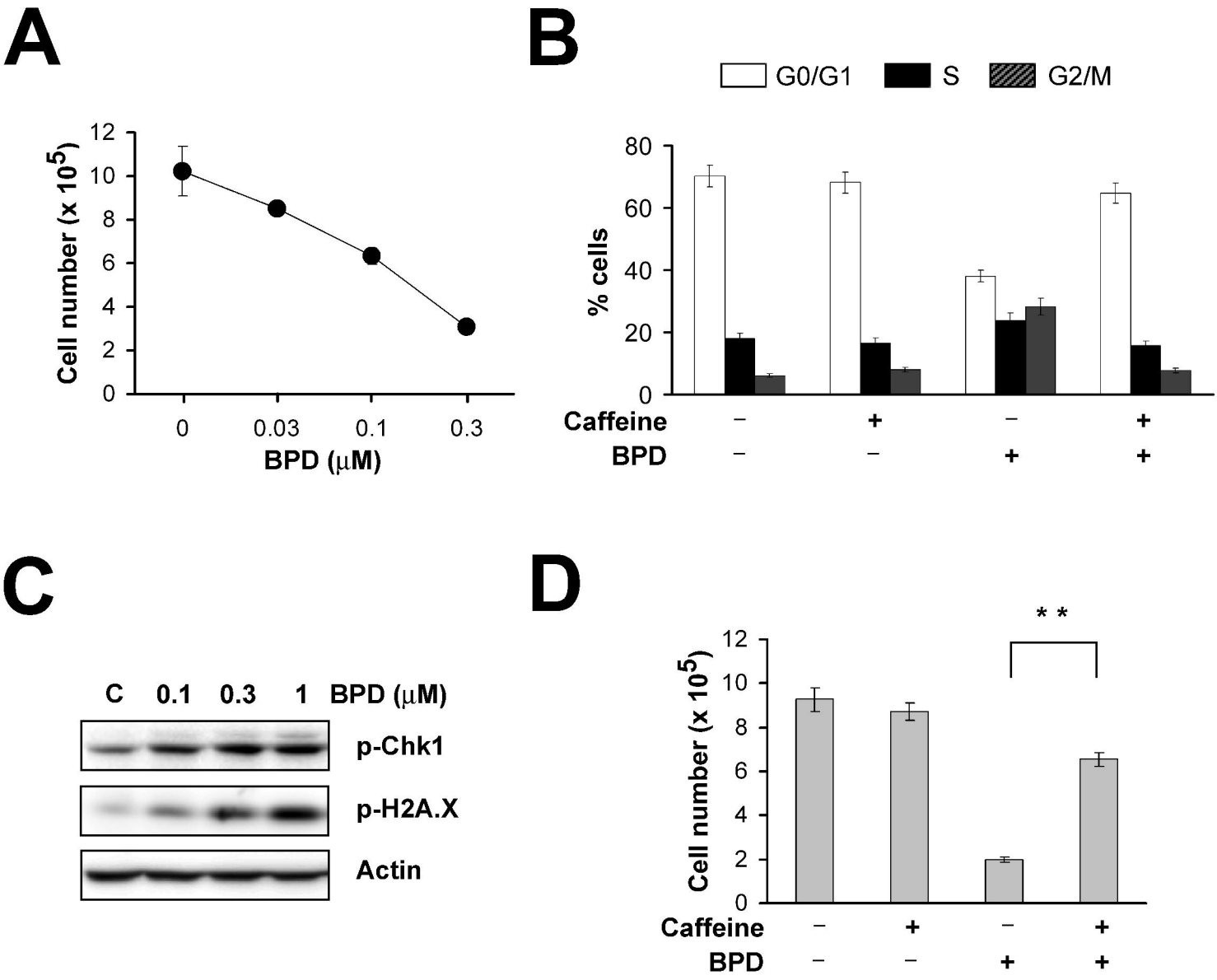
Figure 6


A
Cell number (x 10^5)
12
10
8
6
4
2
0
0
0.03
0.1
0.3
BPD (μM)
B
G0/G1
S
G2/M
% cells
80
60
40
20
0
Caffeine
BPD
C
C
0.1
0.3
1
BPD (μM)
p-Chk1
p-H2A.X
Actin
D
* *
Cell number (x 10^5)
12
10
8
6
4
2
0
Caffeine
BPD