Research Article

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A Src/Abl kinase inhibitor, bosutinib, downregulates and inhibits PARP enzyme and sensitizes cells to the DNA damaging agents

Src/Abl kinaz inhibitörü bosutinib PARP enzim seviyesini azaltıp inhibe eder ve hücreleri DNA hasar verici ajanlara karşı hassaslaştırır

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Abstract

Background: Poly(ADP-ribosyl)ation (PARylation) catalyzed mainly by PARP1 is a highly regulated posttranslational modification associated with several pathways in cellular physiology and genotoxic deoxyribonucleic acid (DNA) damage response. PAR polymers and PARP enzyme function in DNA integrity maintenance and several PARP inhibitors have entered clinical phase studies for cancer therapies.
Material and methods: The effect of bosutinib, a dual Src/Abl kinase inhibitor, on PARylation was fluorometrically measured. The cytotoxic and chemosensitizing effects were assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The levels of DNA repair proteins and PARP enzyme were examined by immunoblotting.
Results: In this study, bosutinib is characterized as a novel PARP inhibitor. Bosutinib inhibited oxidative stress-induced cellular PARylation and nuclear foci formation by downregulating PARP1 levels. Bosutinib was found to be more cytotoxic on Capan1 cells with BRCA2 mutation. Furthermore by acting as a chemosensitizer, bosutinib enhanced the cytotoxicity of doxorubicin (DOXO) and etoposide (ETP) by decreasing phosphorylation of DNA repair enzymes checkpoint kinase 1 (Chk1) and ataxia-telangiectasia mutated (ATM).
Conclusion: By inhibition of both PARP and DNA damage checkpoint kinases, bosutinib increased the phospho-H2AX levels, an early indicator of DNA double strand breaks.

Keywords: PARP inhibitor; PARylation; Bosutinib; Multi-kinase inhibitor; Chemosensitizer.

Özet

Amaç: Başlıca PARP1 enzimi ile katalize edilen poli(ADP-ribozil)asyon (PARilasyon), oldukça sıkı regüle edilen bir posttranslasyonal modifikasyon olup hücresel fizyoloji ve genotoksik DNA hasar yanıtındaki çeşitli yolaklar ile ilişkilendirilmiştir. PAR polimerleri ve PARP enzimi, DNA bütünlüğünün korunmasında fonksiyon göstermektedir ve çeşitli PARP inhibitörleri kanser terapisi için klinik faz çalışmalarına dahil edilmiştir.
Gereç ve Yöntem: Bir Src/Abl kinaz inhibitörü olan bosutinibin PARilasyona etkisi fluorometrik olarak ölçülmüştür. Sitotoksik ve kemohassaslaştırıcı etki, 3-(4,5dimetilmiazoll-2-yl)-2,5-difeniltetrazolyum bromit (MTT) yöntemi ile değerlendirilmiştir. DNA onarım proteinleri ve PARP enzim seviyesi immunoblotlama ile incelenmiştir.
Bulgular: Bu çalışmada, bosutinib yeni bir PARP inhibitörü olarak karakterize edilmiştir. Bosutinib, PARP1

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protein seviyesini azaltarak oksidatif stres ile indüklenmiş hücresel PARilasyonu ve nüklear odak oluşumunu inhibe etmektedir. Bosutinib, BRCA2 mutasyonu içeren Capan1 hücrelerinde daha fazla sitotoksik bulunmuştur. Kemohassaslaştırıcı olarak davranan bosutinib, DNA tamir enzimlerinden Chk1 ve ATM fosforilasyonunu inhibe ederek doksorubisin (DOXO) ve etoposidin (ETP) sitotoksisitesini arttırmaktadır. Hem PARP hem de DNA hasar tamir kinazlarının inhibisyonu ile bosutinib, DNA çift zincir kırıklarının erken bir belirteci olan fosfo-H2AX seviyesini arttırmaktadır.

Sonuç: Bu çalışmadaki verilerimiz bosutinibin bir PARP inhibitörü olarak davrandığını ve kemohassaslaştırıcı etkisinin hem PARP hem de DNA tamir proteinlerinin inhibisyonu sonucu gözlendiğini önermektedir.

Anahtar Kelimeler: PARP inhibitor; PARilasyon; Bosutinib; Multikinaz inhibitörü; Kemohassaslaştırıcı.

Introduction

Deoxyribonucleic acid (DNA) damage occurs both endogenously via replication errors or reactive oxygen species production as byproducts of various metabolic pathways and also exogenously through exposure to chemicals, UV light and radiation. DNA damage repair mechanisms are critical to effectively cope with these damages in order to guarantee accurate cell replication and maintenance of genomic stability. Base excision repair (BER), nucleotide excision repair (NER), and mismatch repair (MMR) are three pathways responsible for resolving single-strand breaks (SSBs); while homologous recombination (HR) and nonhomologous end joining (NHEJ) are to cope with double-stand breaks (DSBs) [1].

One of the main processes that contribute to the DNA repair is the modification of key proteins via Poly(adenosinediphosphate-ribosyl)ation (Poly(ADP-ribosyl)ation, PARylation). PARylation is a complex and highly regulated post-translational modification that plays a critical role in cellular physiology and genotoxic stress response. It regulates pleiotropic events such as maintenance of genomic integrity, telomere maintenance and reinitiation of stalled replication fork [2–4]. Transcription, vesicle trafficking, mitosis, proteostasis, cell cycle, epigenetics, signaling and cell death are also among the physiological processes that are regulated by PARylation [5]. It is noteworthy to mention that the most prominent function of PARylation is the enzymatic reaction during DNA repair.

Poly(ADPribosyl)ating proteins or the Poly(ADP-ribose) polymerase (PARP) superfamily, is a family of 17 proteins having several biological roles including DNA repair, epigenetic regulation, cell cycle regulation, genomic stability maintenance, inflammation, hypoxic response, oncogenic signaling, and cell death [6]. The PARP enzymes function in the post-translational modification of target proteins with ADP-ribose transferred from nicotinamide adenine dinucleotide (NAD^+) molecules. Some of these PARP enzymes like PARP1 and PARP2 are able to catalyze the formation of long, branched poly(ADP-ribose) chains on target proteins; while most of the others transfer mono(ADP-ribose) moiety [6]. Thus, not all PARP enzymes fit the definition of a polymerase and according to a more accurate recent nomenclature PARPs are proposed to be renamed as ADP-ribosyltransferases diphtheria toxin-like (ARTD) [7]. PARP1, the most well-studied member of the family, is an abundant nuclear protein and has the ability to bind DNA via its zinc finger domain. While its basal catalytic activity is low, PARP1 specifically binds to the sites of DNA lesion following DNA damage [6–9]. After binding to the lesion, the conformation of PARP1 is changed; which in turn, increases its catalytic activity. Then PARP1 enzyme becomes active for the addition of PAR moieties on itself and several other proteins that are associated with chromatin, such as histones, p53, topoisomerases and heterogeneous nuclear ribonucleoproteins [8, 9]. The modification of these proteins with short-lived, negatively charged PAR chains relaxes the chromatin structure via electrostatic repulsion so that BER DNA repair pathway members could access the DNA breaks [7–9]. Importantly, high amounts of PAR are only transiently present in the cell as PAR is rapidly hydrolyzed by poly(ADP-ribose) glycohydrolase (PARG) [10]. Besides its cellular benefits in DNA repair, the activation of PARP1 has also been implicated in cell death pathways. When there is excessive DNA damage like in ischemia, PARP1 gets hyperactivated resulting in quick depletion of NAD^+ and ATP and ultimately leads to cell death [11, 12].

Due to their critical role in maintenance of DNA integrity, PAR polymers are attractive chemotherapeutic targets. While the basal levels of PAR in untreated cells are low, DNA-damaging agents can transiently increase PAR levels by 10–500-fold [13]. The PAR level is important for both DNA repair and cell death, two essential paths required for genomic integrity maintenance after DNA damage. PARP1 is responsible for the bulk of cellular PARylation activity especially after binding to DNA breaks [2, 7]. Therefore, developing PARP1 inhibitors (hereafter referred as PARP inhibitors) is one popular area of interest in cancer therapies. Indeed, several PARP inhibitors including olaparib, veliparib and rucaparib have entered human clinical trials. PARP inhibitors are known as radio- and chemo-potentiating agents and sensitize cancer

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cells to cytotoxic agents or radiotherapy that induce DNA damage that would normally be repaired by BER [14–16]. On the other hand, PARP inhibitors are highly selective promising agents in monotherapy of cancer cells with HR deficiencies via generation of chromatid breaks, cell cycle arrest and apoptosis [17, 18]. In this context, PARP inhibition results in synthetic lethality of cells with HR defects [19], such as those harboring mutations on tumor suppressors BRCA1 or BRCA2 [17, 18]. Olaparib in 2014 and rucaparib in 2016 have been Food and Drug Administration (FDA)-approved for BRCA-mutated advanced, previously treated ovarian cancer patients [20]. Interestingly, it is also suggested that PARP inhibition is effective in some tumors that lack DNA repair defects such as HR-proficient HER2-positive breast cancer cells suggesting that PARP might have other functions than its critical role in BER [21].

In one of our recent projects we have screened over 100 different compounds to identify novel PARP inhibitors by measuring inhibition of oxidative stress-induced PAR formation in HeLa cells. Bosutinib (Bosulif, SKI-606, 4-anilino-3-quinolinecarbonitrile), an orally active, second-generation tyrosine kinase inhibitor was found to be a novel PARP inhibitor in our screening. Bosutinib functions as dual kinase inhibitor of Src and Abl kinases. Src is a member of the non-receptor tyrosine kinase family and functions upstream of several critical signaling pathways including PI3K/AKT/mTOR, MAPK/extracellular-signal-regulated kinase (ERK), and JAK/STAT3 signaling. Therefore, Src participates in intracellular signaling pathways and is associated with cell migration, proliferation, and apoptosis [22]. Another non-receptor tyrosine kinase, namely Abl is an oncogene associated with chromosome translocations in human leukemia. Abl translocates within the Bcr gene, which in turn generates Bcr-Abl fusion gene encoding a constitutively active oncogenic tyrosine kinase Bcr-Abl in chronic myelogenous leukemia (CML), [23]. The Bcr-Abl oncoprotein then activates the Ras pathway implicated in aberrant cell proliferation. In addition to inhibiting Src/Abl kinases, bosutinib also targets several other kinases including EGFR, CAMK2G (a Ca^{2+}/calmodulin-dependent protein kinase) and members of the Sterile 20, Tec, and c-Kit kinase families suggesting that bosutinib actually acts as multikinase inhibitor harboring several off-target effects [20, 24]. Besides its FDA-approved usage in CML patients, bosutinib also has anticancer activity in some tumors including breast, prostate, colorectal, cervical cancers and neuroblastoma via Src/Abl signaling in several preclinical studies [25–29].

We report here for the first time that bosutinib harbors PARP inhibitor activity evidenced by several different approaches. Its inhibition activity partly or solely depends on downregulation of PARP1 levels. Furthermore, bosutinib was more cytotoxic on pancreatic adenocarcinoma cells (Capan1), which have defective BRCA2 gene, compared to HR-proficient cervical cancer cells (HeLa) and non-tumoral lung fibroblast (MRC5) cells. We also showed that bosutinib sensitized cells to doxorubicin (DOXO) and etoposide (ETP), where it abolished DOXO- and ETP-induced activation of DNA repair proteins namely, ataxia-telangiectasia mutated (ATM) and checkpoint kinase (Chk)-1 (Chk1) kinases. Furthermore, bosutinib enhanced the phosphorylated H2AX level, which is an indicator of DSBs. To summarize, our data suggests that the chemosensitizer activity of bosutinib is at least partly due to its PARP inhibitor activity.

Materials and methods

Cell culture

Human pancreatic adenocarcinoma Capan1, human endometrial carcinoma HeLa and human lung fibroblasts MRC5 were obtained from American Type Culture Collection and maintained as exponentially growing monolayers by culturing according to the supplier's instructions.

Drug treatments

Olaparib was purchased from Biovision, while DOXO and ETP from Cell Signalling Technology (CST, USA). Bosutinib was obtained from Abcam (UK). Drug stock solutions were prepared in dimethyl sulfoxide (DMSO) and stored in aliquots at –20°C. The final concentration of DMSO did not exceed 0.2% and was administered to the control cells as solvent control.

In experiments where oxidative stress was induced by addition of H_2O_2, cells were first pretreated with bosutinib with serial dilutions (as indicated in Figure 1A, B and C) or 5 μM olaparib for 1 h and then treated with 1 mM H_2O_2 for 10 min. Cells treated with only DMSO were used as a negative control for all experiments.

Required concentrations for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) assay were freshly prepared by diluting the stock solutions in the culture medium. In the application of DNA damage agents, cells were pretreated with 10 μM bosutinib for 2 h and then agents were administered with serial dilutions of DOXO (0.1, 0.5, 1, 2.5, 5 μM) or ETP (10, 20, 30, 40, 50 μM) for 24 h.

Final concentrations of agents were 10 μM for bosutinib, 1 μM for DOXO and 30 μM for ETP in immunoblotting experiments.

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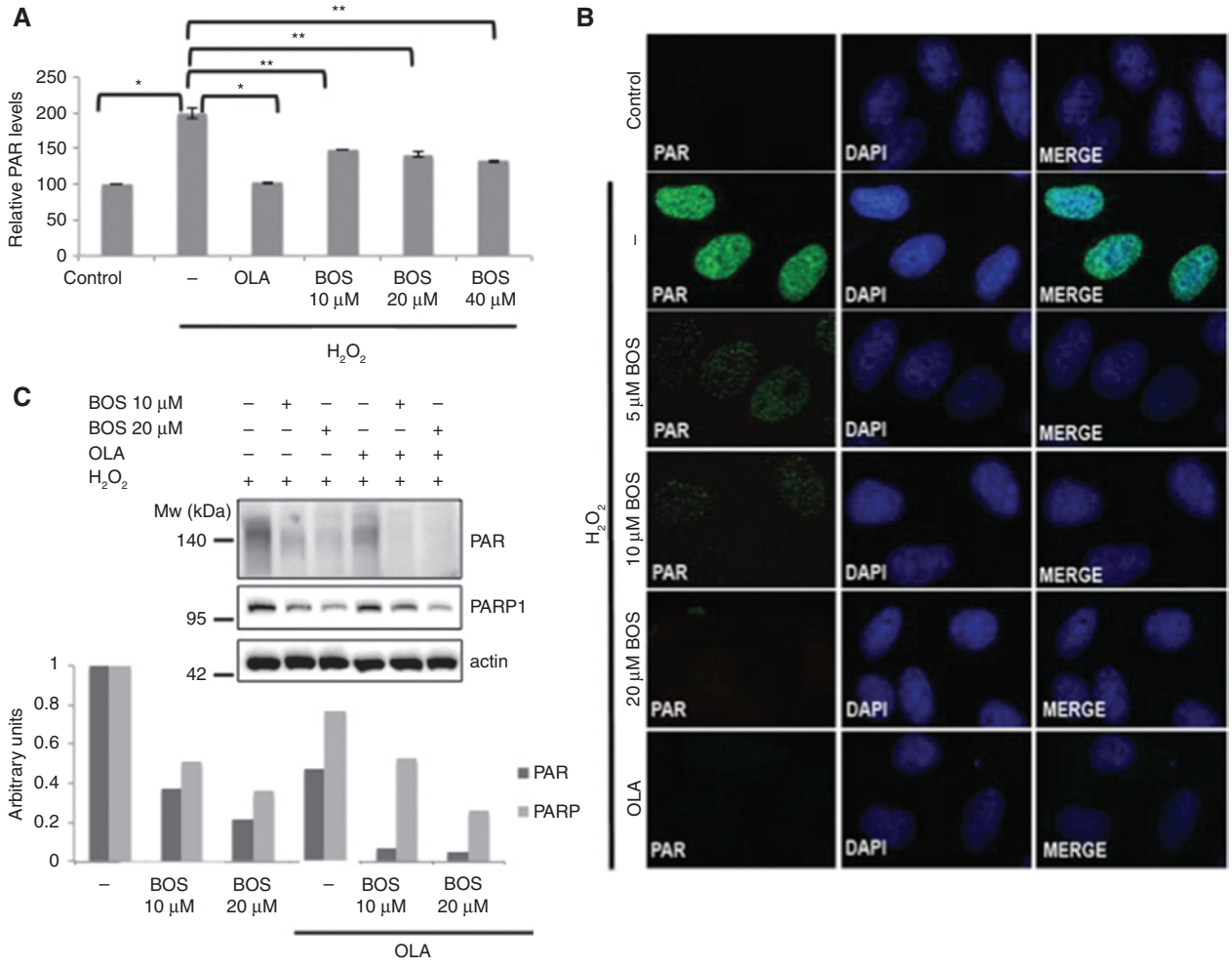


Figure 1: Effect of bosutinib on PARylation and PARP expression.
(A) The effect of bosutinib on cellular PAR levels. HeLa cells were first treated with 10 μM, 20 μM, 40 μM bosutinib (BOS) or 5 μM olaparib (OLA) for 1 h and then treated with 1 mM H_2O_2 for 10 min. Control cells were not treated with H_2O_2. Cellular PAR assay was performed by using anti-PAR antibody and anti-mouse Alexa 488 antibody as indicated in Material and Methods. Cellular PAR levels were measured with a fluorometric multiplate reader and relative PAR levels were presented as graphs through normalization with DAPI signal. Assay was performed by triplicate samples in at least three independent experiments. Significance was determined by two-tailed equal variance the Student's t-test using GraphPad Prism software (*$p \leq 0.0005$; **$p \leq 0.001$). (B) The effect of bosutinib on nuclear foci formation of PARylated proteins. HeLa cells were pretreated with 5 μM, 10 μM, 20 μM bosutinib or 5 μM olaparib for 1 h and then 1 mM H_2O_2 administered for 10 min. Cells were stained with anti-PAR antibody followed by anti-mouse Alexa Flour-488 antibody for foci formation of PARylated proteins and DAPI for the nucleus. Cells were imaged with a 100X objective at identical exposure settings with fluorescence microscope. Immunofluorescence experiments were performed twice, and a representative image was used among at least 15 images taken for each treatment. (C) The reduction of PARylated protein and PARP1 levels by bosutinib. HeLa cells were pretreated with 10 μM, or 20 μM bosutinib or 5 μM olaparib or their combination for 1 h and then 1 mM H_2O_2 administered to the cells. The expression levels of PARylated proteins and PARP1 were determined by immunoblotting. Actin was used as the loading control in all immunoblotting analyses in this study.

MTT assay

The cytotoxic potencies of test compounds were determined by using MTT (Roche, Switzerland) according to the manufacturer's instructions. Cells were seeded into 96-well plate at a density of 7000 cells/well. Next day, cells were treated with compounds and incubated for 24 h at conventional cell culture conditions. The ratio of surviving cells after compound treatment was determined using the MTT assay. The absorbance was measured by using Varioscan microplate reader (Thermo Fisher Scientific, USA) at 570 nm. Experiments were done in triplicates.

Cellular PAR assay

Cellular activity of PARP inhibitors were assessed by measuring the inhibition of the H_2O_2-induced PARylation in HeLa cells. Briefly, 10,000 HeLa cells/well were seeded

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into 96-well black microplates and were allowed to attach to the plate for 24 h. After treatment with either bosutinib or olaparib, DNA damage was provoked by the addition of 1 mM of H_2O_2. Then, cells were fixed by addition of ice-cold methanol-acetone (7:3) and kept at –20°C for 20 min. After repeatedly washing with PBS, the nonspecific binding sites were blocked by incubating cells with 5% bovine serum albumin in PBS–Triton X-100 at 37°C for 30 min. Then samples were incubated first with the primary PAR mAb (Enzo Life Sciences, USA) and then with secondary anti-mouse Alexa Fluor 488 antibody (Thermo Fisher Scientific, USA). Nuclei were stained with the specific nuclear dye 4′,6-diamidino-2-phenylindole (DAPI) (1 µg/mL) for 5 min at room temperature. At the end, fluorescence was read with a fluorometric multiplate reader with (Varioskan, Thermo Fisher Scientific, USA). The obtained PAR signal was normalized with DAPI signal. Experiments were done in triplicates.

Immunostaining

HeLa cells (1×10^5/well) were grown on glass cover slips and treated with bosutinib and H_2O_2. Cells were fixed with ice-cold 100% methanol for 15 min at –20°C. After methanol fixation, cells were rinsed six times with wash buffer and then subsequent standard immunofluorescence analyses were performed using a fluorescence microscope (Olympus IX79, Japan) mounted with 100x apochromat oil objective.

Immunoblotting

After treatments cells were harvested and lysed with RIPA buffer (50 mM Tris-HCl, pH 8.0, 1% NP-40, 0.1% SDS, 150 mM NaCl, 0.1% Triton X-100, 5 mM EDTA) with protease inhibitors (Roche) to prepare whole cell lysates. Protein concentrations were determined by bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific, USA). Equal amounts of proteins were loaded to the gradient precast TGX gels (BIORAD, USA) and proteins were separated by SDS-PAGE electrophoresis using 4–20% gradient gels and transferred to PVDF (Polyvinylidene fluoride) membranes (EMD Millipore, Thermo Fisher Scientific, USA). Membranes were blocked in PBS–0.1% Tween-20 with 5% non-fat dry milk. Mouse monoclonal antibodies used in this study included anti-PAR (Trevigen, USA) (1:1000), anti-actin (Sigma, UK) (1:20,000) and anti-JNK1 (CST, USA) (1:2000). The rabbit polyclonal antibodies used were anti-PARP-1 (CST, USA) (1:1000), anti-ERK1 (Santa Cruz Biotechnology, SCBT, Germany) (1:2000) and anti-phospho-ERK1/2 (Santa Cruz) (1:2000), whereas the rabbit monoclonal antibodies used were anti-phospho-H2AX (CST, USA) (1:1000), anti-phospho-SAPK/JNK (CST, USA) (1:2000), anti-phospho-Chk1 (Cell Signaling) (CST, USA), and anti-phospho-ATM (Abcam, UK) (1:1000). The Horseradish peroxidase (HRP)-coupled secondary antibodies used are goat anti-rabbit and goat anti-mouse (1:5000; Thermo Fisher Scientific, USA). Chemiluminescence signals were detected using Clarity ECL substrate solution (BIORAD, USA) by Fusion-FX7 (Vilber Lourmat, Thermo Fisher Scientific, USA).

Statistics

Data are presented as means ± standard deviation (SD). The statistical significance of differences between groups was assessed by two-tailed equal variance the Student's t-test or two-way ANOVA followed by Bonferroni's post hoc test using Prism (V5, GraphPad Software) and Origin Pro (V8, OriginLab Corp) softwares, respectively. Values of $p < 0.05$ were considered significant.

Results

We utilized a 96 well fluorometric cellular PARylation assay to identify PARP inhibitors among 102 compounds and bosutinib, a dual Src-Abl kinase inhibitor, was found to be one of the novel PARP inhibitors identified in this screening. As seen in Figure 1A, treating HeLa cells with H_2O_2 increased total cellular PAR levels, which is consistent with previous studies suggesting that H_2O_2 treatment induced PARP1 activity [30, 31]. When cells were pretreated with bosutinib 1 h prior to the addition of H_2O_2, cellular PAR levels were significantly diminished suggesting that bosutinib inhibits H_2O_2-induced PARP activity similar to the well-known PARP inhibitor olaparib (Figure 1A). Consistently, the expression of PARylated proteins seen as loci were increased in the nucleus after H_2O_2 exposure, whereas pretreating cells either with olaparib or bosutinib dramatically decreased these nuclear foci (Figure 1B). Next, we analyzed the effect of cotreatment of bosutinib and olaparib on the cellular PAR and PARP levels using immunoblotting. While pretreatment of cells with bosutinib abolished the H_2O_2-induced PAR levels, combination of bosutinib and olaparib caused further reduction of the PAR levels, suggesting a synergy between bosutinib and olaparib on PARP-inhibition (Figure 1C). Interestingly, we observed that bosutinib treatment dramatically decreased

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PARP levels in a dose-dependent manner (Figure 1C). This data indicates that bosutinib downregulates PARP protein levels via acting through a different mechanism than olaparib in PARP inhibition.

To test the antiproliferative effect of bosutinib, increasing concentrations of bosutinib was used on HeLa, Capan1 and MRC5 cells for 24 h. The results suggest that cell viability was significantly diminished in a dose-dependent manner (Figure 2). Consistent with the enhanced sensitivity of PARP inhibitors in the HR-defective cells we observed that bosutinib is more cytotoxic on Capan1 cell line which is defective in BRCA2 [32] compared to HR-proficient HeLa cancer cell line and non-tumoral MRC5 fibroblast cell line (Figure 2).

Bosutinib I (Bos-I), also known as the "authentic" bosutinib is an isomer, which functions as a chemosensitizer [20]. To test whether bosutinib sensitizes the cells to DNA damaging agents, we treated HeLa cells with increasing concentrations of DOXO or ETP in combination with 10 μM bosutinib for 24 h. We found that the combination of bosutinib with DOXO or ETP showed greater cytotoxic effect when compared to either agent alone, suggesting that the cytotoxic effects of DOXO and ETP is enhanced with the addition of bosutinib in HeLa cells (Figure 3). Besides PARP inhibitors, some of the kinase inhibitors such as DNA damage checkpoint inhibitors have been also reported to harbor chemosensitization activity. Likewise, Bos-I is suggested to have off-target effects such as abrogation of the DNA damage checkpoints by inhibiting of Chk1 and Wee kinases [20]. Therefore, to further investigate

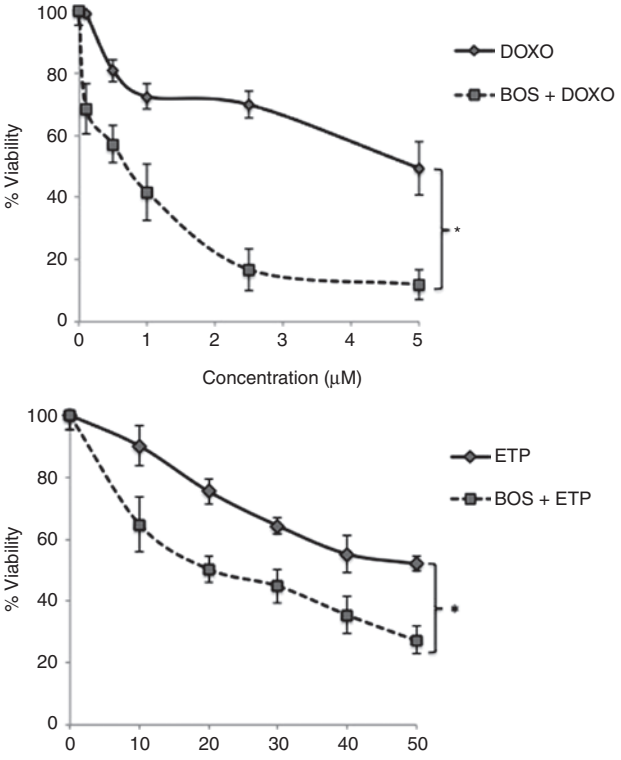


Figure 3: Sensitization of cells to the DNA damaging agents by bosutinib.
HeLa cells pretreated with 10 μM bosutinib for 2 h followed by addition of increasing concentrations of DOXO (0.1, 0.5, 1, 2.5, 5 μM) or ETP (10, 20, 30, 40, 50 μM) were incubated for 24 h and the viability of cells were assessed by MTT assay as in Figure 2. The MTT assay was performed by triplicate samples in at least three independent experiments. Two-way ANOVA was used to determine the significance of the differences. *$p \leq 0.05$ (ANOVA).

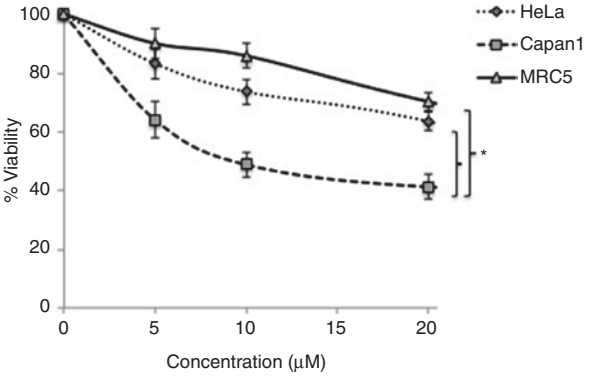


Figure 2: The cytotoxic effect of bosutinib on Capan1, HeLa and MRC5 cells.
Effects of bosutinib on proliferation of HeLa, Capan1 and MRC5 cells were assessed by MTT assay for 24 h. The absorbance of cells treated with DMSO as solvent control was considered as 100% and cell viability of the applied concentrations was calculated. The MTT assay was performed by triplicate samples in at least three independent experiments. Two-way ANOVA was used to determine the significance of the differences. *$p \leq 0.05$ (ANOVA).

the mechanism of chemosensitization by bosutinib, we investigated the effect of combining bosutinib with DOXO or ETP on the phosphorylation/activation levels of DNA repair proteins. Treatment of HeLa cells with DOXO or ETP induced the DNA damage checkpoint that was reflected in the activation of Chk1 and ATM measured via their phosphorylation status. The addition of bosutinib abolished phosphorylation of Chk1 and ATM (Figure 4). Besides their role in checkpoint control, ATM and Chk1 are required for both maintaining the stability and reinitiation of stalled replication forks [20]. Likewise PARP enzymes and PARylation also regulate reinitiation of stalled replication forks [19] and inhibition of PARP induces DNA SSBs, which increases the formation of DSBs after the stalling DNA replication forks [33]. Therefore, inhibition of either DNA damage checkpoint kinases or PARP enzyme results in the collapse of stalled forks creating DSBs. In this context, we evaluated the level of phospho-H2AX, by which DSBs are detectable. When cells were treated with either by DOXO

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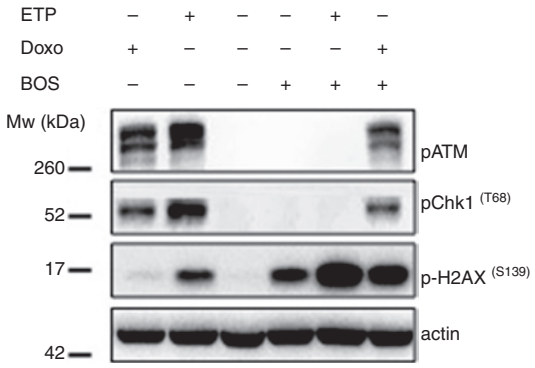


Figure 4: The effect of bosutinib on DNA damage response proteins. HeLa cells were pretreated with 10 μM bosutinib for 2 h and then DOXO or ETP were administered to the cells for 24 h at a concentration of 1 μM and 30 μM, respectively. Cells were lysed to prepare whole cell lysates and the levels of DNA damage checkpoint kinases (ATM, Chk1) and phospho-H2AX were investigated by immunoblotting.

or ETP, phospho-H2AX levels were increased. Notably, co-treatment of bosutinib with these two agents further increased phospho-H2AX levels (Figure 4). To sum up, our results suggest that bosutinib sensitizes the cells and enhances DNA damage induced by DOXO and ETP, as implied by increases in phosphorylated H2AX levels via abrogation of both PARylation and Chk1/ATM DNA damage checkpoint kinases.

Discussion

There are several areas of targeted therapies with recently increased interest including targeting the metabolism of PAR biopolymers. After DNA damage, PARP enzymes, primarily PARP1, synthesize PAR [21]. PAR biopolymers have very critical cellular functions; therefore the levels of these polymers are tightly regulated via their short half-life by catabolism through the highly specific enzyme PARG [10]. The metabolism of PAR is very important for both efficient DNA repair or cell death [21]. There are several PARP inhibitors that are in clinical trials with high efficacy in cancer cells with HR deficiency, such as those with BRCA gene mutations. These cancer cells are very dependent on PARP-mediated repair for survival. Therefore, the accumulation of SSBs in DNA by PARP inhibitor treatment eventually leads to DSBs after encountering by replication forks, which would not be repaired in the absence of BRCA, resulting in cell death [32, 33]. On the other hand, PARP inhibitors are also known as potentiators of chemo- and radiotherapies by enhancing DNA DSBs after chemo- and radiotherapy. PARP inhibitors exhibit their activity by either blocking the catalytic activity or trapping PARP on the damaged DNA which in turn blocks the recruitment of downstream repair proteins [34]. Interestingly, it has been suggested that the inhibition of PARP1 by olaparib is more cytotoxic than PARP1 silencing suggesting that there are multiple roles of PARP inhibitors [34].

In an effort to identify new PARP inhibitors, we screened over 100 compounds utilizing a fluorometric cellular PARylation assay measuring the inhibition of oxidative stress-induced PAR levels. One of the hits from this screening was bosutinib a serine/threonine kinase inhibitor. Even though bosutinib is mainly known as a dual Src-Abl kinase inhibitor, it is actually a multikinase inhibitor having several targets including EGFR and Ca^{2+}/calmodulin-dependent protein kinase [20, 24]. Furthermore, bosutinib's isomer, Bos-I, was reported to have some off-target effects such as abrogation of DNA damage checkpoint pathway by inhibiting Chk1 and Wee kinases [20].

Capan1 cell line with BRCA2 mutation is a human tumor cell line commonly used for evaluating the cytotoxicity of PARP inhibitors as monotherapy in vitro [32]. Our results suggest that this cell line is clearly very sensitive to bosutinib (Figure 2) compared to HeLa cervical cancer cells with intact HR pathway and nontumoral MRC5 fibroblasts. After verifying the PARP inhibitor activity of bosutinib in HeLa cells (Figure 1), we further explored the molecular mechanism of this inhibition and we found that bosutinib significantly downregulated PARP1 protein levels (Figure 1C). PARP1 activity is also known to be regulated by various endogenous factors such as xanthines, purines, Vitamin D and estrogen [35–37]. The inhibition of Jun N-terminal kinase1 (JNK1) suppresses H_2O_2 induced-PARP1 activation in mouse embryonic fibroblasts suggesting that JNK1 mediates H_2O_2-induced cell death via sustained PARP1 activation [31]. Similarly, PARP1 activity is also blocked by ERK1/2 inhibitors in neuronal cultures and astrocytes [38]. Both JNK1 and ERK1/2 regulate PARP activity through its phosphorylation [31, 38]. On the other hand, bosutinib has been shown to inhibit ERK1/2 in neuroblastoma cells and another multikinase inhibitor sorafenib was suggested to inhibit JNK1 in hepatocellular carcinoma cells [29, 39]. Since we detected the PARP inhibitor activity of bosutinib in HeLa cells, we hypothesized that PARP phosphorylation might be inhibited by bosutinib either directly or indirectly. Consistent with this idea, bosutinib inhibited both ERK1/2 and JNK in HeLa cells, which are one of the major upstream regulators of PARP via phosphorylation (Supplemental Figure 1). To sum up, our data suggests that bosutinib might inhibit PARP via two different mechanisms; downregulating PARP protein level and/or inhibiting PARP phosphorylation, which in turn inhibits PARP activation.

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Previous studies have shown that PARP inhibitors sensitize tumor cells treated with DNA damaging agents. Authentic Bos-I is reported to have some off-target effects on the DNA repair pathway [20]. Thus we tested the chemopotentialization effect of bosutinib when it is combined with DOXO or ETP. As expected, bosutinib showed synergistic activity with both DNA damaging agents used in this study. When we checked the activity of DNA repair proteins we found that DOXO- or ETP-induced activation of DNA repair proteins ATM and Chk1 were diminished by bosutinib. Our results are consistent with one recent report suggesting that authentic Bos-I inhibits Chk1 in PANC1 cells [20]. Interestingly, our data suggests that Bosutinib has more significant effect on phosphorylation of ATM and Chk1 when cotreated with ETP compared to data obtained from cotreatment with DOXO. This might be due to the applied concentrations of ETP and DOXO in our assay system. One other explanation might be the possible distinct activity mechanism of ETP and DOXO. Even though both ETP and DOXO are topoisomerase II inhibitors, there are several studies suggesting topoisomerase II independent cell death caused by DOXO including free radical formation, ceramide overproduction etc., [40]. Further studies are required to identify the molecular reason behind this observation. On the other hand, Bosutinib might have diminished the activity of ATM both through its activity as kinase inhibitor and/or its PARP inhibition activity, since PAR has been shown to be necessary for the full activation of ATM [41]. We also further assessed DNA damage by measuring the levels of phospho-H2AX, an early response marker for DSB signaling. Our results suggest that bosutinib augmented DOXO- or ETP-induced levels of phospho-H2AX.

Consequently, bosutinib might be a strong anticancer drug candidate targeting several pathways of tumorigenesis via its main and multiple off-target effects. This study, to the best of our knowledge, is the first study that demonstrates the PARP inhibitor activity of bosutinib. However, further studies are required to identify whether there is a direct effect of bosutinib on the phosphorylation levels of PARP, which is known to regulate its catalytic activity. Furthermore, characterization of the mechanism of PARP downregulation would be critical especially to investigate PARP regulation systems.

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Conflict of interest statement: The authors declare that they have no conflict of interest.

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