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Chk1 inhibitor synergizes quinacrine mediated apoptosis in breast cancer cells by compromising the base excision repair cascade

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ABSTRACT

Quinacrine (QC) causes apoptosis in breast cancer cells by induction of DNA damage, arrest of cells in S-phase, and by topoisomerase inhibition. Here, we show that QC-mediated apoptosis is not only due to increased DNA damage but also by compromising cell cycle checkpoints and base excision repair (BER) capacity in breast cancer cells. QC decreased CHK1, CDKs (CDC2, MDM2, CDC6), cyclins (B1, E1) and CDC25-A in a dose dependent manner. The expression of basal ATR remains unaltered but pATR (Ser-428) increased after QC treatment. A *CHK1 inhibitor*, SB218078, was also tested alone and in combination with QC. Like QC, SB218078 caused apoptosis by DNA damage and S-phase arrest. The combination of QC and SB218078 increased apoptosis by blocking the cell cycle in G2/M, which caused a mitotic catastrophe, and induced DNA damage at a higher level in comparison to individual compound treatments. Both drugs individually or in combination decreased the levels of replication protein A (RPA). Measurement of the expression of BER (SP- and LP-BER) proteins and direct *in vivo* BER activity revealed that the QC/SB218078 combination caused apoptosis in cancer cells by disrupting the induction of BER, which represents a novel means of potentially treating breast cancer.

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1. Introduction

Quinacrine (QC, 9-amino acridine) has been shown to inhibit the growth of several types of cancer cells and is currently in phase II clinical trials. Recently, we have investigated the mechanism of action for the anticancer activity of QC in several studies. For example, a systematic study using genetically modified HCT116 colon cancer cell lines showed that QC mediated autophagy and apoptosis depends on p53 and p21 [1]. Inhibition of the Wnt-TCF signaling cascade by QC or the topoisomerase poison etoposide depends on the Adenomatous Polyposis Coli (APC) protein [2]. We have also observed an induction of p53 and p21, S-phase arrest, DNA damage, and inhibition of topoisomerase activity [3]. The induction of p53 by DNA damage is well known to cause cell cycle arrest and provide time for DNA repair, or induce apoptosis if the damage is considered irreparable [4,5]. For QC, the mechanism of the S-phase arrest and DNA damage response is not understood.

Eukaryotic cells activate cell cycle check points in response to DNA damage through complex kinase signaling networks that prevent cell cycle progression [6,7]. Checkpoint signaling also mediates the recruitment of DNA damage/repair proteins to sites of damage [8]. The DNA damage response (DDR) pathway is broadly categorized into two major kinase signaling branches. The ATM/CHK2 branch is thought to be primarily activated after DNA double strand breaks (DSBs), while the ATR/CHK1 branch responds to stalled replication forks associated with DNA single strand breaks (SSBs) or bulky lesions [9]. Replication stress is increasingly being acknowledged as an important source of genome instability that drives tumorigenesis [10,11]. CHK1 has been shown to be essential for normal replication and its inhibition by UCN-01 causes aberrant replication, ATR activation, and DNA breaks [12]. Studies using a dominant negative ATR mutant showed that the ATR/CHK1 pathway plays an important role in modulating the S-phase checkpoint induced by topoisomerase 1 inhibitors such as camptothecin [13,14]. Several reports suggest that the combination of a topoisomerase inhibitor and Chk1 inhibitor increases the sensitivity of cancer cells [15,16]. This includes the CHK1 inhibitor SB218078, which enhanced the cytotoxicity of topotecan and camptothecin in HeLa and HT-29 cell lines [15,17].

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Replication protein A (RPA) is a heterotrimeric, single strand DNA-binding protein essential for DNA replication and repair [18]. Hyper phosphorylation of the 32 kDa subunit of RPA by ATR/CHK1 is well known to occur in response to replication stress in particular [19–21]. RPA has also been implicated in repair processes including base excision repair (BER), specifically long-patch BER mediated by flap endonuclease (FEN-1) and PCNA [5,22–24]. Several studies have shown that cells lacking functional BER components such as XRCC1 are also hypersensitive to topoisomerase-I poisons [25,26]. Moreover, XRCC1 is typically recruited to sites of DNA single strand breaks by PARP1 and polyADP-ribose (pADPr) [27,28]. These studies have led to proposed models that PARP1 contributes to repair of topoisomerase-I mediated damage by recruiting a multiprotein complex consisting of TDP1, XRCC1, DNA ligase III, and PNKP to DNA sites in which topoisomerase-I is trapped in a covalent complex by an inhibitor, or strand breaks that result from replication through and/or repair of such trapped covalent complexes [29–31]. In other words, there is a plausible link between the DNA damage caused by topoisomerase poisons, replication stress signaling, and BER.

In the present study, we investigated the involvement of ATR/CHK1 signaling in QC induced S-phase arrest, DNA damage, BER capacity, and antiproliferative activity in breast cancer cells. QC induces S-phase checkpoint signaling via ATR/CHK1 similar to topoisomerase-I poisons. QC induces DNA damage and also disrupts BER. We show that the CHK1 inhibitor SB218078 synergistically increased QC-mediated cytotoxicity in breast cancer cells by increasing DNA damage and cell death. Interestingly, co-incubation of SB218078 and QC abrogated the S-phase arrest and resulted in a G2/M arrest to accompany the heightened sensitivity to QC. This suggests that the loss of checkpoint signaling during replication stress prevents proper repair, which manifests in a G2/M arrest and cell death from the resulting unrepaired damage during replication.

2. Materials and methods

2.1. Maintenance and treatment of cell lines

The human breast cancer cell lines, MCF-7 (ATCC cat # HTB-22), ZR75-1 (ATCC cat # CRL-1500), MDA-MB-231 (ATCC cat # HTB-26) and T47D (ATCC cat # HTB-133), were cultured and maintained in Dulbecco's modified Eagle's medium as described by Preet et al. [3]. The normal breast epithelial cells, MCF-10A (ATCC cat # CRL-10317) were grown and cultured in DMEM-F12 according to the protocol mentioned earlier [3]. The cell culture reagents were procured from HIMEDIA, Mumbai, India. QC (Cat # Q3251), and other reagents were purchased from Sigma Chemical Co. (St Louis, MO) and were dissolved in dimethyl sulfoxide. SB218078 (Cat # 559402) was purchased from Calbiochem (Merck KGaA, Darmstadt, Germany). The anti-ATR (#MA1-231580) was purchased from Affinity BioReagents, CO, USA, anti-CHK 2 (sc #17747), anti-GAPDH (sc #25778), anti-CDC 6 (sc #9964) anti-DNA Ligase III (sc-166374) were procured from Santa Cruz Biotechnology, Inc., CA, USA. The anti-MDM2 (AB16895), anti-Pol ε (#93H3A) and anti-XRCC-1 (AB47920) were purchased from Abcam, MA, USA, anti-Pol β (NB #600-1025) was purchased from Novus Biologicals, CO, USA. The anti-CHK 1 (#2345), anti-pCHK1 (#2344S), anti-WRN (#4666), anti-pγH2AX (#9718P), anti-PhosphoH3 (#3642), anti-CBP (#7389), anti-p-ATR (#2853S), anti-CDC2 (#9116), anti-pCDC2 (#9111S), anti-CDC 25A (#3652), anti-CYCLIN B1 (#4135), anti-CYCLIN E (#4129), anti-APE (#4128), anti-PARP (#9542), anti-FEN1 (#2746), anti Bcl-XL (#2764), anti-BAX (#2772), anti-RPA (#2267), anti-DNA PK (#4602), anti-PCNA (#2586) were procured from Cell Signaling Technology, MA, USA. Cells were treated with individual compounds as described in the text and respective figure legends. In the combination treatment experiments, cells were first exposed to a fixed concentration (0.75 μM) of SB218078 for 12 h, after which the media containing SB218078 were replaced with fresh media containing different concentrations of QC, then further incubated for 24 h.

2.2. Western blot analysis

Cells were treated with QC, SB218078, and their combination. After drug treatment, western blotting was carried out according to protocols described previously [3]. The level of GAPDH expression was used as internal control for equal protein loading. Densitometric analysis of each independent blot was done using a gel documentation system (UVP Gel Doc-It 310, Cambridge, UK). The relative fold change was calculated with respect to the control.

2.3. Immunocytochemistry analysis of p-γH2AX (Ser-139) expression

Breast cancer cells were grown on coverslips and treated with indicated concentrations of QC, SB218078, and in combination for 24 h. Then cells were washed with 1X PBS and fixed with acetone: methanol in a 1:1 ratio for 20 min at −20 °C followed by blocking in 2% BSA and 0.02% triton X-100 in 1X PBS for 3 h at 4 °C. The cells were washed once with 1× PBS and incubated with p-γH2AX (Ser-139) (cat # 9718 from Cell Signalling) antibody for 2 h at 4 °C. Unbound antibodies were removed by washing twice with 1X PBS. Secondary antibody conjugated to FITC was added and the cells were incubated for 1 h at 4 °C. The cells were then washed thrice in 1X PBS and the nuclei were counter stained with DAPI to visualize nuclei. Images were captured under fluorescence microscope (Nikon, Tokyo, Japan) at 40× magnification.

2.4. Cell cycle analysis

Cell cycle distribution was measured by FACS analysis. Briefly, 1×10^6 cells were seeded in a 6-well plate and allowed to adhere overnight. Cells were treated with concentrations of SB218078 as shown in figures ranging from 0 to 2 μM for 24 h in fresh medium. After treatment, cells were harvested using 0.05% trypsin, washed twice in ice-cold 1X PBS and fixed with chilled 70% ethanol for 2 h at −20 °C. The cells were again washed with 1X PBS and then treated with 80 mg/ml RNase A and 50 mg/ml propidium iodide for 2 h. The stained cells were analyzed using a FACS Canto II flow cytometer (Becton and Dickinson, CA, USA).

2.5. Alkaline comet assay

Alkaline comet assay was performed to evaluate the DNA damage efficacy of the drug according to the protocol described in Preet et al. [3]. Approximately 1×10^5 MCF-7 cells were seeded in 24-well tissue-culture plates and were exposed to various concentrations of SB218078 for 24 h. In a separate set of experiments, the MCF-7 and MDA-MB-231 cells were treated with a fixed concentration of QC (5 μM), SB218078 (0.75 μM) and a combination of QC (5 μM) and SB218078 (0.75 μM) respectively for 24 h. The slides were processed and stained with SYBR green and the migration of DNA was observed using a fluorescence microscope (Nikon, Japan) at 10× magnification. The comet tail lengths were analyzed by TriTek CometScore™ software (Tritek Corporation, VA, USA).

2.6. MTT cell viability assay

To measure the anchorage dependent cell viability of different breast cancer cells along with the normal breast epithelial cells, MTT assays were carried out according to the protocol mentioned

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