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SHORT REPORT

Effects of the novel DNA dependent protein kinase inhibitor, IC486241, on the DNA damage response to doxorubicin and cisplatin in breast cancer cells

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Summary The purpose of this study was to determine the degree to which the novel DNA-PKcs inhibitor, IC486241 (ICC), synergizes the cytotoxicity of DNA damaging agents in 3 genetically diverse breast cancer cell lines. The sulforhodamine B (SRB) assay was employed as a primary screening method to determine the *in-vitro* cytotoxicity and the degree of synergy of ICC in combination with the topoisomerase II inhibitor, doxorubicin, or the DNA cross linking agent, cisplatin. Molecular mechanisms underlying drug toxicity were probed using immunostaining and flow cytometry, as well as, the alkaline comet assay to detect DNA damage. In this study, improved cytotoxicity and significant synergy were observed with both anticancer agents in the presence of nontoxic concentrations of ICC. Moreover, ICC decreased doxorubicin-induced DNA-PKcs autophosphorylation on Ser2056 and increased doxorubicin-induced DNA fragmentation. In conclusion, the novel DNA-PKcs inhibitor, ICC, synergistically sensitized 3 breast cancer cell lines to doxorubicin and cisplatin. Enhanced efficacy of doxorubicin was achieved by inhibiting non-homologous end joining resulting in increased accumulation of DNA damage.

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Introduction

Conventional chemotherapy for breast cancer often employs DNA damaging drugs to prevent proliferation and stimulate apoptosis of cancer cells. One class of chemotherapeutic agents with excellent activity in treatment of metastatic breast cancer is the anthracycline group, of which doxorubicin is a member [1]. Cisplatin, another effective agent against metastatic breast cancer is a DNA cross-linking agent. Both cisplatin and doxorubicin can result in the formation of highly cytotoxic double strand breaks (DSBs). Cisplatin damages DNA by forming DNA adducts that result in interstrand and intrastrand cross-links. Such cross-links left unresolved can result in DSBs. Although the exact mechanism of DSB formation is unclear, it is believed that cross-links distort the shape of the DNA double helix resulting in DNA damage during gene expression and replication [2]. In contrast, doxorubicin's cytotoxicity is mediated by inhibiting the function of topoisomerase II resulting in the formation of DSBs [3].

Two major mechanisms are used by cells to repair DSBs: homologous recombination (HRR) and non-homologous end joining (NHEJ). The heterotrimeric serine/threonine kinase DNA-PK complex plays a major role in coordinating NHEJ processes by recognizing and binding to DSB sites and recruiting other repair proteins. NHEJ is initiated when the Ku70/Ku80 heterodimer (non-enzymatic component of DNA-PK) comes in contact with DNA ends at DSB sites. The DNA bound Ku complex then recruits and activates other proteins involved in end joining including DNA-PKcs (enzymatic component of DNA-PK), polymerase μ, polymerase λ and the ligase IV/XRCC4/XLF complex [4, 5]. The importance of DNA-PK in DSB repair is illustrated by studies showing that inhibition of DNA-PKcs sensitizes breast cancer cells to ionizing radiation [6]. Moreover, down regulation of DNA-

PKcs by siRNA in MCF-7 breast cancer cells sensitizes the cells to cisplatin [7]. Furthermore, the specific DNA-PKcs inhibitors, Nu7026 and Nu7441, sensitize leukemia cells to anthracyclines and fludarabine [8, 9].

To improve therapeutic efficacy a new generation of DNA-PK inhibitors has been developed. These specific DNA-PK inhibitors (containing an arylmorpholine substructure (Fig. 1)) have better pharmacokinetic profiles than other specific DNA-PK inhibitors, are relatively nontoxic in mice and enhance the efficacy of ionizing radiation *in-vitro* and *in-vivo* [10]. On testing these compounds (IC compounds including IC486241 (ICC)) *in vitro* we observed synergistic sensitization of colon cancer cell lines to irinotecan by ICC at 1–2 μM [which are obtainable concentrations in mice (unpublished data, Luitpold Corp)] [11]. Also as part of the aforementioned colon cancer study, flow cytometric data indicated reduced phosphorylation of DNA-PKcs and comet assay studies showed increased DNA damage with SN38 in combination with DNA-PK inhibitors when compared to cells treated with SN38 alone.

Given these results and previous investigations with DNA-PKcs inhibitors and DNA-PKcs specific siRNAs we speculated that inhibition of DNA-PKcs would be effective in synergizing treatment of breast cancer cell lines with DNA damaging agents. The sulforhodamine-B (SRB) assay was used to measure the cytotoxicity of two DNA damaging drugs, cisplatin and doxorubicin, and combinations of these drugs with a novel DNA-PKcs inhibitor, ICC. Multiple single/combinations of agents were tested for cytotoxicity in various breast cancer cell lines (MCF7, BT-20 and MDA-MB-436). Multiple breast cancer cell lines were used as a model system to give a broad view of drug effects in a variety of genetic backgrounds. MCF7 cells are estrogen/progesterone receptor positive while both BT-20 and MDA-MB-436 lack these hormone receptors. These three cell lines are her-2 negative. Only MDA-MB-436 is BRCA1 mutated and p53 mutated [12, 13]. We hypothesized that treatment of these three cell lines with doxorubicin or cisplatin in combination with the DNA-PKcs inhibitor ICC would synergize the cytotoxic effects of these drugs.

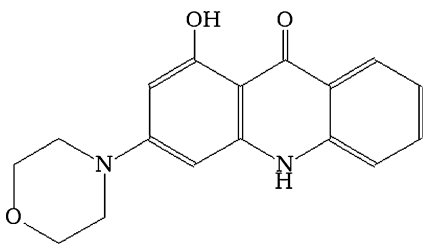

Fig. 1 Molecular structure of the DNA-PKcs inhibitor IC486241

Materials and methods

Cell culture and reagents

MCF7, BT-20 and MDA-MB-436 breast cancer cell lines were obtained from the American Type Culture Collection and were maintained at 37°C in 5% CO_2 and RPMI with 10% fetal bovine serum and 1% penicillin/streptomycin. Chemicals and reagents were obtained from Sigma-Aldrich or Invitrogen. IC486241 (ICC) was kindly provided by Luitpold Pharmaceuticals.

Sulforhodamine (SRB) cytotoxicity assays

SRB assays were performed according to the method of Vichai et al. 2006 [14]. In this assay SRB stain binds to basic amino acid moieties under mildly acidic conditions facilitating total protein quantification and by implication, cell density determination. The assay is amenable to high throughput screening, is linear over a 20 fold range of cell numbers and has sensitivity similar to fluorescence based assays making it an ideal tool for cytotoxicity studies [14]. Briefly, cells were seeded at low density (final density within the linear range of the assay) in 96 well culture dishes and incubated overnight. Cells were subsequently treated with cisplatin or doxorubicin alone, the DNA-PKcs inhibitor alone (ICC), or combinations of cisplatin or doxorubicin and ICC (concentrations indicated in results). Five days post drug treatment cells were fixed with trichloroacetic acid, stained with SRB, and analyzed for percent growth on a 96 well plate reader. Efficacies of the various drug treatments were determined by calculating 50% inhibitory concentrations (IC_{50}) and synergy values. Synergy values (I value) were calculated using the equation of Berenbaum [15] as previously used in our laboratory [11, 16]. Using this equation I values less than 1 indicate synergy, equal to 1 indicate additive behavior, and greater than 1 indicate inhibitory drug interactions.

Comet assays

Alkaline comet assays were performed according to the method of Olive & Banàth 2006 [17] as previously used in our laboratory [11, 18]. Cells were treated with doxorubicin alone, ICC alone or in combination with doxorubicin. Twenty-four hours post treatment cells were harvested and subjected to single cell gel electrophoresis in 1% low melt agarose gels. Gels were dried and stained with propidium iodide and subsequently individual cells were photographed at 100× magnification and analyzed using Comet Assay IV software (Perceptive Instruments, UK). Average Olive-tail-moments were calculated from the staining intensity to tail length of at least 50 comets. Increased tail length and

increased DNA in the tail region indicate increased DNA damage.

Flow cytometric analyses

These experiments were performed according to the protocol of Amrein et al. 2007 [16]. Briefly, cells were treated with drugs as for the comet assay and analyzed for cell cycle distribution (stained with 5 μg/mL 7AAD and 0.2 mg/mL RNAse-A), pDNA-PKcs (anti-phospho-Ser2056, anti-phospho-Thr2609, Abcam, Cambridge MA) and γH2AX (anti-phospho-Ser139, Upstate, Lake Placid NY). The fluorescence intensity of individual cells was measured by flow cytometry and presented as histograms and as mean overall fluorescence divided by the mean overall fluorescence of the DMSO control. The presented data is representative of 3 replicate experiments.

Analysis

There were at least 5 replicates for all SRB experiments and at least 3 replicates for all other experiments. Comet assays and cell cycle analysis were repeated 3 times. Means were calculated and then compared employing the Students *T*-test analysis ($p \leq 0.05$) using Graphpad incorporated's "Quickclacs" software.

Results

SRB cytotoxicity assays (Table 1) showed that in comparison to the other 2 breast cancer cell lines the MDA-MB-436 cell line was 7–8 fold more sensitive to cisplatin consistent with its known BRCA1 mutated status [13]. Furthermore, synergism was observed between ICC and each of the primary drugs tested (Table 1). The IC_{50} values of cisplatin and doxorubicin for each cell line were significantly reduced when used in combination with ICC (Table 1). While 5 μM concentrations of ICC yielded the lowest I values (greatest synergy), this concentration of ICC was somewhat cytotoxic when used alone (final cell density was 62%, 61%, and 65% of control for BT20, MCF7, and MDA breast cancer cells respectively). Lower concentrations of ICC (1–2 μM) were nontoxic to all cell lines tested and yet reduced the IC_{50} values of both cisplatin and doxorubicin (Table 1). Furthermore, the DNA-PK inhibitor at 1, 2 and 5 μM concentrations produced significant synergy with cisplatin and doxorubicin in the three breast cancer cell lines tested. Similar levels of synergy were observed in all cell lines in spite of the variable characteristics of the cell lines (ER/PR, BRCA and p53). In addition, the level of synergy was ICC dose dependent with increasing concentrations of ICC causing increased drug synergy.

To examine the effects of the combined drug treatment (ICC with doxorubicin) at the molecular level, flow cytometry was used to determine phosphorylation of DNA-PK on serine 2056 (Ser2056), threonine 2609 (Thr2609) and H2AX on serine-139 (γH2AX), as well as, cell cycle status in BT-20 cells 24 h post drug treatment. For these studies the BT-20 cell line was chosen as representative as similar levels of synergy were observed for all cell lines and drug combinations tested. Nu7026, a known specific inhibitor of DNA-PKcs was used to gauge the potency of ICC [9]. Cell cycle analysis of BT-20 cells 24 h post treatment showed accumulation of cells in the G2/M phase of the cell cycle after treatment with doxorubicin or doxorubicin in combination with 2 μM ICC or Nu7026 (Fig. 2). As expected, cells treated with ICC or Nu7026 showed lower phosphorylation of DNA-PKcs,

Table 1 Fifty percent inhibitory concentrations (IC_{50}) and synergy values for various drug combinations (I) were determined using the SRB assay. Synergy was determined using the equation $I = a/A + b/B$ where a = IC_{50} of the anticancer drug with IC486241 (ICC), A = IC_{50} of anticancer drug alone, b = concentration of ICC in combination with the anticancer drug, and B = IC_{50} of ICC alone. I > 1 indicates antagonistic drug interaction, I = 1 indicates additive behavior and I < 1 implies a synergistic drug interaction. cis = cisplatin dox = doxorubicin

Breast cancer cell line	BT-20		MCF7		MDA-MB-436	
Treatment	IC50 μM	I value	IC50 μM	I value	IC50 μM	I value
ICC	25.0±7.00		11.0±2.20		34.0±10.0	
cis	1.67±0.43		1.60±0.36		0.22±0.09	
cis+1 μM ICC	1.30±0.26	0.76±0.08	0.93±0.21	0.75±0.08	0.18±0.04	0.67±0.14
cis+2 μM ICC	0.81±0.28	0.52±0.18	0.86±0.23	0.75±0.12	0.17±0.01	0.69±0.05
cis+5 μM ICC	0.64±0.30	0.62±0.20	0.32±0.16	0.65±0.11	0.07±0.03	0.49±0.08
dox	0.09±0.01		0.07±0.01		0.13±0.02	
dox+1 μM ICC	0.06±0.01	0.76±0.13	0.04±0.02	0.66±0.23	0.10±0.02	0.84±0.15
dox+2 μM ICC	0.04±0.01	0.58±0.15	0.03±0.01	0.54±0.12	0.07±0.01	0.61±0.09
dox+5 μM ICC	0.02±0.01	0.37±0.07	0.02±0.01	0.61±0.18	0.03±0.01	0.38±0.10

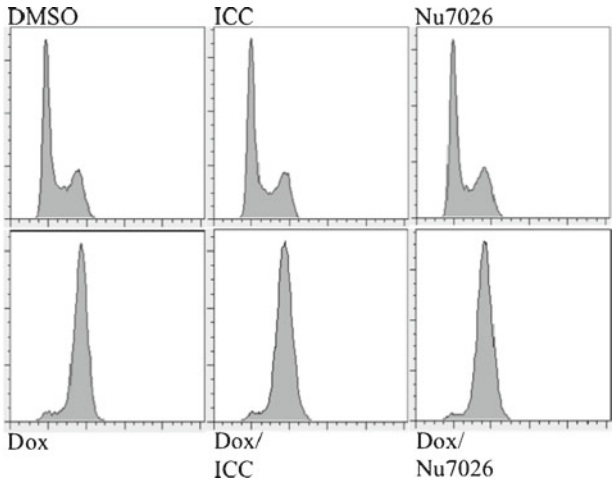

Fig. 2 Cell cycle analysis of BT-20 cells 24 h after treatment with doxorubicin (0.09 μM) or the combination of doxorubicin (0.09 μM) and IC486241 (ICC) (2 μM) or Nu7026 (2 μM). Tabulated results are displayed as a percentage of total events, determined by flow cytometry

Treatment	G0/G1	S	G2/M
DMSO	60.77±1.22	6.89±0.15	32.30±1.25
ICC	63.03±0.06	6.30±0.17	31.67±2.47
Nu7026	58.87±1.31	6.35±0.22	34.80±1.18
Doxorubicin	4.27±0.60	1.92±0.22	93.53±0.64
Doxorubicin/ICC	3.91±0.51	2.25±0.23	93.43±0.97
Doxorubicin/Nu7026	3.13±0.77	1.49±0.46	94.97±1.24

particularly on Ser2056, as compared to the DMSO treated control. DNA-PKcs phosphorylation in doxorubicin/ICC or doxorubicin/Nu7026 treated samples was decreased compared to the levels observed in samples treated with doxorubicin alone (Fig. 3). In addition, doxorubicin treatment was associated with a dramatic increase in γH2AX and addition of 2 μM ICC or 2 μM Nu7026 to 0.09 μM doxorubicin treatment diminished this response (Fig. 3).

To examine the amount of DNA damage with a more direct technique, the alkaline comet assay was performed. Figure 4

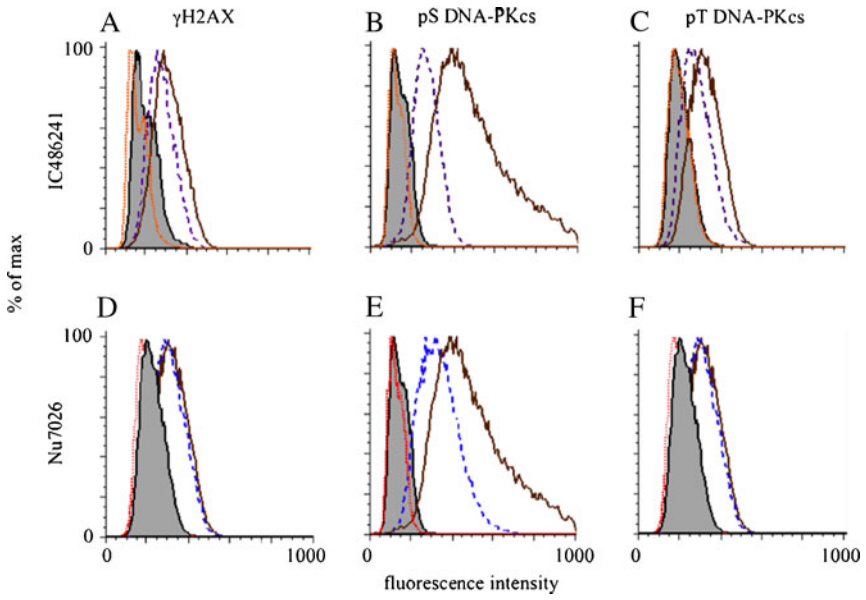

Fig. 3 Flow cytometric analysis of BT-20 breast cancer cells treated for 24 h with DMSO (*grey shaded area*), IC486241 (ICC) (*orange dotted line* (**a**, **b**, **c**)), Nu7026 (*red dotted line*, (**d**, **e**, **f**)), doxorubicin alone (*brown solid line*), or doxorubicin in combination with IC486241 (ICC) (purple dashed line (**a**, **b**, **c**)) or Nu7026 (*blue dashed line* (**d**, **e**, **f**)). The summary table shows the mean fluorescence intensity of all events for each treatment divided by the mean fluorescence intensity of the DMSO control

Summary Table

	IC486241	Nu7026	Dox	Dox/ICC	Dox/Nu7026
pT DNA-PK	0.87	0.90	1.43	1.25	1.38
pS DNA-PK	0.90	0.90	3.48	1.88	2.36
γH2AX	0.82	0.82	1.57	1.40	1.47

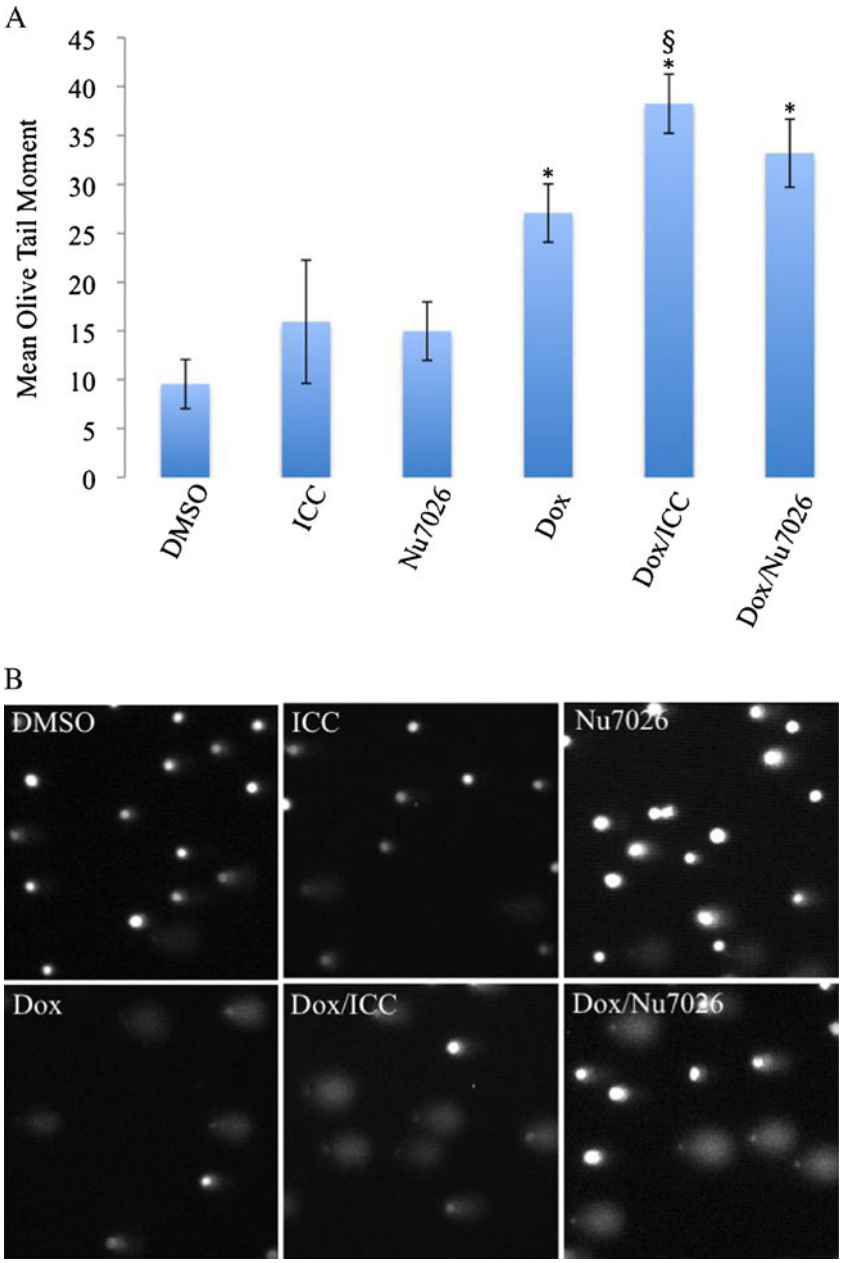

Fig. 4 **a** Average Olive tail moment of BT-20 cells treated for 24 h with doxorubicin or the combination of doxorubicin (Dox) (0.09 μM) and IC486241 (ICC) (2 μM) or Nu7026 (2 μM). **b** Representative comets for each treatment. * = significantly different from DMSO treatment, $p \leq 0.01$; § = significantly different from Dox $p \leq 0.05$

shows that treatment of cells with doxorubicin alone and in combination with ICC produced significantly larger comets than the DMSO control and the combination treatment produced comets larger than those of doxorubicin alone.

Discussion

Cytotoxicity of doxorubicin and cisplatin is synergistically increased by the DNA-PK inhibitor ICC

The main goal of this study was to determine if inhibition of the NHEJ DNA repair pathway with a novel DNA-PKcs inhibitor synergizes killing of breast cancer cells with DNA damaging drugs. Significant synergy was observed with both cisplatin and doxorubicin in combination with the DNA-PKcs inhibitor, ICC. More importantly, synergy was observed at nontoxic drug concentrations (1–2 μM) of ICC. Results of the work described in this report support the concept that inhibition of DNA repair can be highly cytotoxic in the presence of anticancer agents. Here we show that inhibition of NHEJ greatly enhances the effective dose of the anticancer drugs, cisplatin and doxorubicin, in 3 diverse breast cancer cell lines. To our knowledge this is the first report of specific DNA-PKcs inhibitors sensitizing breast cancer cells to doxorubicin.

Inhibition of DNA-PK and enhanced DNA damage underlie ICC synergy

In response to DNA damage, the NHEJ repair pathway is activated when the serine/threonine kinase DNA-PKcs complex recognizes and binds to DSB sites. After binding to broken DNA ends, the DNA-PK complex facilitates recruitment of other repair proteins involved in processing, alignment and ligation of the DNA ends [19–24]. An important step in this process is phosphorylation/autophosphorylation of DNA-PKcs [25]. The small molecule inhibitor ICC, like Nu7026, reversibly inhibits this critical step by blocking the ATP binding pocket of DNA-PKcs thus preventing autophosphorylation and phosphorylation of other DNA-PKcs substrates [9, 26, 27]. In this work, treatment with the DNA damaging drug doxorubicin caused significant phosphorylation of DNA-PKcs and this phosphorylation was reduced when a DNA-PKcs inhibitor was added simultaneously with the DNA damaging agent. This demonstrates that: (a) DNA-PKcs is phosphorylated in response to DNA damage and (b) this response is inhibited in the presence of ICC. Also, increased γH2AX staining and increased comet scores after doxorubicin treatment suggests that DNA damage is the mechanism underlying the cytotoxicity of this drug. Furthermore, greater comet scores with the combination treatment of doxorubicin and ICC suggest increased DNA damage probably due to inhibition of DNA damage repair. DNA-PKcs phosphorylation/autophosphorylation is required for efficient repair of DNA damage, particularly DSBs. Interestingly, treatment of BT-20 cells with the combination of ICC and doxorubicin showed a decrease in γH2AX compared to doxorubicin alone. Two possible explanations for this observation are: (a) doxorubicin in combination with ICC produces less DSBs or (b) the addition of a DNA-PK inhibitor prevents DNA-PK from phosphorylating H2AX. Comet assay data suggest that DSBs are in fact increased by the addition of ICC to the doxorubicin treatment. This implies that inhibiting DNA-PK in the presence of DNA damage not only reduces autophosphorylation but also affects phosphorylation of other substrates such as H2AX [28]. Additionally, the observed cell cycle arrest following treatment with doxorubicin or doxorubicin combined with ICC indicates that inability to progress in the cell cycle may be an important factor in doxorubicin-induced cytotoxicity.

In conclusion, the cytotoxicity of the anticancer drugs, doxorubicin and cisplatin, in breast cancer cell lines with varied genetic backgrounds was enhanced by combined treatment with the novel small molecule DNA-PK inhibitor, ICC. At nontoxic concentrations, ICC showed synergistic behavior with the anticancer drugs doxorubicin and cisplatin. Mechanistically, the increased cytotoxicity of doxorubicin in combination with ICC was the result of decreased DNA-PKcs activity and increased DNA damage resulting from inhibition of the NHEJ pathway. This work demonstrates for the first time synergism between a novel DNA-PK inhibitor ICC and the DNA damaging agent, doxorubicin, in breast cancer cell lines.

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