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DNA damage response (DDR) pathway engagement in cisplatin radiosensitization of non-small cell lung cancer

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ABSTRACT

Non-small cell lung cancers (NSCLC) are commonly treated with a platinum-based chemotherapy such as cisplatin (CDDP) in combination with ionizing radiation (IR). Although clinical trials have demonstrated that the combination of CDDP and IR appear to be synergistic in terms of therapeutic efficacy, the mechanism of synergism remains largely uncharacterized. We investigated the role of the DNA damage response (DDR) in CDDP radiosensitization using two NSCLC cell lines. Using clonogenic survival assays, we determined that the cooperative cytotoxicity of CDDP and IR treatment is sequence dependent, requiring administration of CDDP prior to IR (CDDP-IR). We identified and interrogated the unique time and agent-dependent activation of the DDR in NSCLC cells treated with cisplatin-IR combination therapy. Compared to treatment with CDDP or IR alone, CDDP-IR combination treatment led to persistence of γH2Ax foci, a marker of DNA double-strand breaks (DSB), for up to 24 h after treatment. Interestingly, pharmacologic inhibition of DDR sensor kinases revealed the persistence of γ-H2Ax foci in CDDP-IR treated cells is independent of kinase activation. Taken together, our data suggest that delayed repair of DSBs in NSCLC cells treated with CDDP-IR contributes to CDDP radiosensitization and that alterations of the DDR pathways by inhibition of specific DDR kinases can augment CDDP-IR cytotoxicity by a complementary mechanism.
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1. Introduction

More than 200,000 people will be diagnosed with lung cancer in the United States this year, accounting for greater than 25% of all cancer deaths [1]. Non-small cell lung carcinomas (NSCLC) are the most common lung cancers and are typically diagnosed at an advanced stage, having spread beyond the primary tumor site. Since at this stage curative surgical options are often limited [2], treatment of locally advanced disease typically includes administration of a platinum-containing drug, such as cisplatin (*cis*-diamminedichloroplatinum II; CDDP) and ionizing radiation [IR] [3,4]. Treatment with a combination of both CDDP and IR improves survival over either treatment alone with the greatest survival observed with concomitant rather than sequential treatment. [5–8]. However, cancer model systems developed to investigate combination CDDP-IR treatment have yielded varying results, including reports of potential antagonistic interactions that are inconsistent with the clinical data [9,10]. Therefore, a better understanding for the observed CDDP-IR clinical synergy is important.

Covalent binding of CDDP to DNA forms intra- and inter-strand DNA adducts which distort the double helical configuration. The DNA-CDDP intra-strand adducts are repaired by the nucleotide excision repair (NER) pathway while inter-strand adducts are repaired by the homologous recombination repair (HRR) pathway, and hypersensitivity to CDDP is often observed in cells deficient in either NER or HRR [11–14]. IR causes DNA nucleotide modifications, single and double strand DNA breaks (DSBs), both directly and indirectly via formation of oxygen free radicals. DSBs are particularly

Abbreviations: NSCLC, non-small cell lung cancer; CDDP, cisplatin; IR, ionizing radiation; CDDP-IR, combination treatment with cisplatin followed by ionizing radiation; DDR, DNA damage response; DSB, double strand break; NER, nucleotide excision repair; HRR, homologous recombination repair; NHEJ, non-homologous end-joining; Gy, Gray.

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toxic to the cell, as a single DSB has been demonstrated to trigger cell death [15]. IR-induced DSBs are repaired predominantly by the non-homologous end-joining (NHEJ) pathway, and NHEJ deficient cancer cells are hypersensitive to IR [16,17]. DNA damage caused by both CDDP and IR activates DNA damage response (DDR) cascades which coordinate a complex interaction of downstream pathways to determine cell fate, including coordination of DNA repair, cell cycle arrest and apoptosis. The DDR is initiated at the site of DNA damage by the early (sensor) protein kinases: ataxia telangiectasia mutated (ATM), ATM and Rad3-related (ATR) and DNA-PKcs. While there is some overlap, ATM is primarily involved in the DDR to DSBs, such as those created by IR. DNA DSBs can be characterized by the detection of γ-H2Ax foci; downstream effectors of the DDR pathway which have been observed to correlate directly to the number of DSBs and persistence of which correlates with cellular survival [18–20]. ATR is important in the DDR to single strand breaks, which are felt to develop on CDDP-damaged DNA through replication stress [21]. Impaired function of ATM or DNA-PKcs leads to radiosensitization while inhibition of ATR has been shown to sensitize some cells to CDDP [12,22–27].

The cooperative interaction of CDDP and IR is dependent on CDDP repair, as cells deficient in NER or HRR show increased radiosensitization to CDDP [9,17,28,29]. The presence of a CDDP lesion on DNA inhibits NHEJ [17,30,31] and we hypothesize that CDDP-IR synergy is determined by a CDDP lesion at close proximity to a DSB. However, despite the recognition of a likely role for DNA repair pathways in CDDP radiosensitization, little is known about the actual mechanism and role of the DDR in radiosensitization. This mechanism is of paramount importance, as drugs specifically targeting the DDR are currently under investigation in pre-clinical and early clinical trials. Here we investigate the impact of the DDR in CDDP-IR co-treatment in NSCLC. Our study supports a role for retained DSBs in CDDP radiosensitization and identifies a dissociation of DDR sensor kinase activation from sustained DSBs.

2. Materials and methods

2.1. Materials

Compounds and reagents were purchased from Thermo-Fisher Scientific (Waltham, MA), unless otherwise stated.

2.2. Antibodies

Antibodies were obtained from the following commercial sources: anti-H2Ax^{Ser139} (Millipore; clone JBW301), anti-P53BP1^{Ser25} (Novus Biologicals; NB100-1803), anti-53BP1 (ThermoFisher Scientific; PA1-16565), anti-pChk1^{S345}, anti-pChk2^{T68}, anti-Chk1, anti-Chk2, anti-ATM (Cell Signaling Technology; 2348S, 2661S, 2G1D5, 1C12 and D2E2 respectively), anti-pATM^{S1981} (Rockland, NC9306342), anti-β-actin (Life Technologies, clone AC-15) and anti-vinculin (Abcam, clone ab18058).

2.3. Cell culture and treatment

Two NSCLC cell lines, A549 (CCL-185) and H460 (HTB-177), were obtained from the American Type Culture Collection and verified via STR testing (Manassas, VA). H460 and A549 cells were grown as previously described and incubated at 37 °C in a humidified 5% CO_2 atmosphere [30]. Cisplatin (Sigma) was added at the indicated concentrations to complete medium for 2 h at 37 °C. Following incubation with CDDP, cells were washed three times with PBS and replaced with fresh media lacking CDDP. Media was replaced with PBS prior to IR or mock IR treatments. For experiments using NU7441 (Tocris Bioscience), KU-55933 (Tocris Bioscience) or VE-821 (MedChem Express), cells were incubated with vehicle (DMSO) and/or the respective inhibitors at the concentrations listed (total of 0.1% DMSO) for 2 h prior to treatment with IR. Incubation with the drug was continued until cell processing (0.5 or 24 h) or for 24 h after IR treatment (clonogenic survival assays).

IR treatments (Figs. 1–5) were performed on ice using an HP Faxitron series X-ray generator (Faxitron Bioptics LLC). X-rays were filtered through a 0.5 mm aluminum filter at 160 kV resulting in a dose rate of 2.5 Gy/min. IR treatments (Fig. 6) were performed in ice using a Precision X-RAD 320 X-ray generator (Precision X-Ray). X-rays were filtered using a 0.5 mm aluminum filter at 160 kV delivering 0.737 Gy/min. The devices undergo routine maintenance with dosimetry testing.

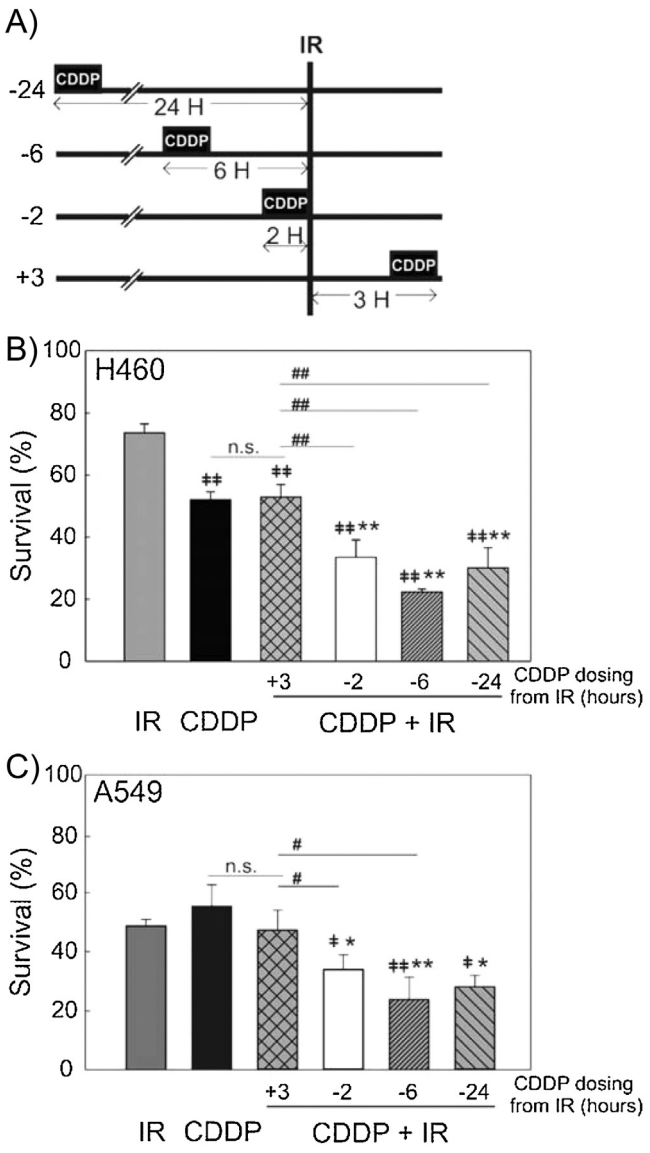

Fig. 1. Differential Radiosensitization is Dependent on Time from Cisplatin Treatment: A schematic shows the treatments and time-course used to determine survival with CDDP-IR combination treatment (A). Clonogenic survival assays were used to determine cell survival in H460 (B) and A549 (C) NSCLC cells treated with CDDP (4 μM, 2 h) at various time intervals from IR. Treatment with CDDP was 2, 6 or 24 h before (−2, −6, −24) or 3 h after (+3) IR treatment. N = 3, mean ± SD. *p < 0.05, **p < 0.001 compared to CDDP. ‡p < 0.05, ‡‡p < 0.001 compared to IR. #p < 0.05, -p<0.001 compared to +3.