1. INTRODUCTION

Colorectal cancer (CRC) is one of the major causes of cancer-linked mortality worldwide. Synthetic lethality is a pragmatic targeted cancer therapy in which cancer cell-specific vulnerabilities such as genetic defects/somatic mutations are exploited for selective cancer therapy by targeting genetic interactors (synthetic lethal interactors) of such mutation/defects present in cancer cells. In this study, we investigated the synthetic lethal interaction between checkpoint kinase 2 (CHEK2) and peroxiredoxin-2 (PRDX2) in CRC cells to precisely target CRC cells having CHEK2 defects. We have performed siRNA-mediated silencing and n-carbamoyl alanine (NCA)-mediated inhibition of PRDX2 in CHEK2-null HCT116 cells to confirm the synthetic lethal (SL) interaction between PRDX2 and CHEK2 as the cell population reduced significantly after silencing/inhibition of PRDX2. Additionally, treatment with NCA resulted in an increased level of total ROS in both cell types (HCT116 and CHEK2-null HCT116 cells), which further confirms that inhibition of PRDX2 results in an increased ROS level, which are mainly responsible for DNA double-strand breaks (DSBs). ROS-induced DNA DSBs get repaired in HCT116 cells, in which CHEK2 is in the normal functional state, but these DNA DSBs persist in CHEK2-null HCT116 cells as confirmed by the immunofluorescence analysis of 53BP1 and γ-H2AX. Finally, CHEK2-null HCT116 cells undergo apoptosis due to persistent DNA damage as confirmed by immunofluorescence analysis of cleaved caspase-3. The findings of this study suggest that PRDX2 has a SL interaction with CHEK2, and this interaction can be exploited for the targeted cancer therapy using NCA as a drug inhibitor of PRDX2 for the therapy of colorectal cancer having CHEK2 defects. Further studies are warranted to confirm the interaction in the preclinical model.
mutated genes in cancer play a role in the DNA repair mechanism such as the homologous recombination repair (HRR) pathway (also called as the error-free DNA repair pathway). The HRR mechanism is very crucial for the integrity of the cells as it is involved in the repair of DNA double-strand breaks (DSBs).\textsuperscript{13,14} Till date, the synthetic lethal interaction between BRCA1/2 and PARP1 is the best studied, and here also, BRCA1/2 also plays an important role in the HRR pathway. It is the only synthetic lethal interaction that reaches the clinical trials, and efficacy of olaparib (PARP1 inhibitor) therapy was found to be significant in breast and ovarian cancer patients having defects in BRCA1/2.\textsuperscript{15,16} The checkpoint kinase 2 (CHEK2) gene is responsible for the maintenance of chromosomal stability as CHEK2 is involved in the HRR pathway to repair DNA DSBs, and it also regulates cell cycle arrest and cell death mechanisms.\textsuperscript{17,18} Moreover, somatic mutations in the CHEK2 gene has been reported in CRC and many other cancers.\textsuperscript{19,20} Figure S1 In light of the aforementioned facts, CHEK2 is an excellent cancer cell-specific vulnerable candidate to be exploited for the synthetic lethal approach. The peroxiredoxin-2 (PRDX2) enzyme is responsible for the conversion of hydrogen peroxide (H$_2$O$_2$) to water (H$_2$O) in the detoxification pathway, as H$_2$O$_2$ is well-known to induce DNA damage especially DNA DSBs.\textsuperscript{21−23} It is reported that prdx2 (PRDX2 in human) has a synthetic lethal interaction with dun1 (CHEK2 in human) in the yeast model.\textsuperscript{24} According to an earlier report, some interactions are evolutionarily conserved from simple unicellular organisms to complex multicellular ones.\textsuperscript{25} Therefore, synthetic lethal interactions between PRDX2 and CHEK2 expected to be present in cancer cells also as a detoxification pathway are highly conserved.

In this study, we investigated the synthetic lethal interaction between CHEK2 and PRDX2 to precisely target CRC cells having CHEK2 defects. The rationale for the hypothesis is that inhibition or downregulation of PRDX2 leads to enhanced levels of H$_2$O$_2$, which cause DNA DSBs. CHEK2 is responsible for the repair of DNA DSBs as it is involved in the HRR pathway. In normal cells, wild-type CHEK2 will be functional and repair the DNA DSBs, while CRC cells having defects in CHEK2 will be unable to properly repair DNA DSBs and expected to follow an alternative repair pathway, i.e., nonhomologous end joining (NHEJ), which is an error-prone mechanism and leads to accretion of lethal DNA modifications and ultimately cell death.\textsuperscript{26}

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents. Primary antibodies such as PRDX2 (LFMA0144, Invitrogen), CHEK2 (MAS15416, Invitrogen), S3BP1 (PA116665, Invitrogen), GAPDH (ITTS052, Immunotag), γ-HAX (PAS28778, Invitrogen), and cleaved caspase-3 (PSA16335, Invitrogen) were used in this study. We procured and used the following secondary antibodies: (i) goat anti-rabbit IGG-HRP-conjugated secondary antibody (656120, Invitrogen), (ii) anti-rabbit IgG (H + L), F(ab')2 fragment (Alexa Fluor 555 Conjugate) (4413, Cell Signaling technology), and (iii) goat anti-rabbit IgG (H + L) cross-adsorbed secondary antibody, Alexa Fluor 488 (A-11008, Invitrogen). siRNAs for PRDX2 and GAPDH were purchased from Ambion (Invitrogen, Thermo Scientific). Lipofectamine was purchased from Invitrogen (Thermo Scientific). An Amplex red hydrogen peroxide/peroxidase assay kit was purchased from Invitrogen (Thermo Scientific). MTT (with 98% purity), fetal bovine serum (FBS), trypsin (with 98% purity), PBS, glycerol (with 98.2% purity), Triton X-100 (99%), Bradford reagent, and paraformaldehyde (with 99% purity) were obtained from Hi-Media Labs. CHEK2-proficient HCT116 human colorectal cancer cells were purchased from NCCS, Pune. CHEK2-knockout HCT116 cells were given as a kind gift by Dr. Bert Vogelstein from John Hopkins University.

2.2. Cell Culture. HCT116 cells and CHEK2-null HCT116 cells were grown in McCoy’s 5A media with 7−10% FBS under suitable cell culture conditions such as 5% CO$_2$ at 37 °C and at 95% humidity inside a CO$_2$ incubator. All cell lines were grown to check for bacterial or fungal contamination, cell growth, and cellular morphology.

2.3. Small Interfering RNA (siRNA)-Mediated Transient Transfection. Briefly, cells were seeded in a 6-well plate, and after 24 h, when cells reached about 70% confluence, they were treated with siRNA (75 nM) for the different target genes such as PRDX2 and GAPDH. After the siRNA treatment, cells were scraped from the cell culture flask and processed for protein isolation and western blotting to confirm gene silencing. GAPDH was used as an internal control. For siRNA-mediated gene silencing experiments, siRNAs were mixed with lipofectamine for targeting different genes.

2.4. High-Content Cellular Imaging. A Cytation-5 cell imaging multimode reader (BioTek Instruments) was used to image the cells. Briefly, 10,000 cells were seeded in a 96-well plate, and after 24 h, cells were treated with siPRDX2 and $n$-carbamoyl alanine (NCA)—a PRDX2 inhibitor.\textsuperscript{27} Cell fixation was done using paraformaldehyde, and subsequently, Hoechst was used to stain the nucleus. Imaging was performed using a 4X objective, and Gen-5 software was used for nuclear counting.

2.5. Cytotoxicity Assay. MTT assay was performed to assess the efficacy of NCA against HCT116 and CHEK2-null HCT116 cells by the method described earlier with some modifications.\textsuperscript{26} Briefly, 10,000 cells were seeded in a 96-well plate. After 24 h, both HCT116 and CHEK2-null HCT116 cells were treated with different concentrations of NCA starting from 1 nM to 1 M with a 10-fold increment, and cells were allowed to grow for 72 h. Then, MTT assay was performed, and the dose−response curve was plotted, and EC$_{50}$ values and % cell viability were calculated using GraphPad Prism.

2.6. DCFDA Assay for the Detection of ROS Generation. Both HCT116 and CHEK2-null HCT116 cells were treated with 2',7'-dichlorofluorescein (DCFH). In response to intracellular reactive oxygen species (ROS), DCFH is converted to the fluorescent product (DCF). After treatment with DMSO (control) and NCA, both cell types were trypsinized, collected, and washed using serum-free McCoy’s 5A media. Cells were then treated with DCFH for 10 min in the dark and then treated with DAPI. The fluorescent product (DCF) was detected using fluorescence microscopy.

2.7. Dihydroethidium (DHE) Assay for the Detection of Cellular Superoxide Anion Radical Generation. HCT116 and CHEK2-null HCT116 cells were seeded onto a sterile coverslip. After 24 h, cells were treated with DMSO (control) and NCA and were then trypsinized, collected, and washed using serum-free McCoy’s 5A media and subsequently treated with 10 μM dihydroethidium (DHE) (in FBS-free media) and incubated in the dark for half an hour at 37 °C in a CO$_2$ incubator. After incubation, cells were washed with PBS and imaged using a fluorescence microscope.
2.8. Amplex Red Assay for the Detection of Cellular H₂O₂ Generation. The concentration of H₂O₂ was determined according to the manufacturer’s protocol. HCT116 and CHEK2-null HCT116 cells were treated with DMSO (control) and NCA. Cells were then put into the RIPA buffer for 30 min in a refrigerator and centrifuged at 20,000g for 30 min at 4 °C. For the detection of H₂O₂, an equal volume (50 μL) of supernatant and working solution (Amplex Red-10μM, HRP-0.2 U/mL in sodium phosphate buffer, pH 7.4) was mixed and incubated at room temperature for half an hour. The concentration of H₂O₂ was detected spectrophotometrically by measuring the absorbance at 560 nm, and the cellular H₂O₂ concentration was calculated by extrapolating the absorbance against the H₂O₂ standard curve (1–5 μM/L).

2.9. Immunofluorescence. To investigate the effects of NCA against HCT116 and CHEK2-null HCT116 cells, we investigated two biomarkers such as S3BP1 and γ-H₂AX (DNA double-strand break marker) and cleaved caspase-3 (apoptosis marker). Briefly, cells (1 × 10⁶) were grown in a sterile 6-well plate onto poly-l-lysine-coated coverslips for 24 h. Both HCT116 and CHEK2-null HCT116 cells were treated with DMSO (control) and NCA. After 24 h, cells were washed with PBS, fixed with 4% paraformaldehyde for 30 min, and then again washed three times with PBS. Subsequently, cellular permeabilization was done using 0.1% Triton X-100 in PBS for 10 min and then incubated in blocking buffer (5% BSA in PBS + 0.1% Tween 20) for 30 min. After blocking, cells were treated with anti-γ-H₂AX, anti-S3BP1, and anticleaved caspase-3 primary antibodies overnight at 4 °C and then washed with PBS and secondary antibodies such as goat anti-rabbit IgG (H + L) cross-adsorbed secondary antibody, Alexa Fluor 488 (for cleaved caspase-3) and anti-rabbit IgG (H + L), F(ab’)+ L fragment (Alexa Fluor 555 conjugate) (for γ-H₂AX and S3BP1) were added for 1 h at room temperature, washed with washing buffer, and stained with DAPI for nuclear staining. Cells were washed with PBS, mounted, and imaged using a 40× objective of an inverted fluorescent microscope (Zeiss Axio Vert.A1).

2.10. Protein Estimation. Protein estimation was done using the Bradford reagent to detect the protein concentration. Bovine serum albumin was used as a standard, and the absorbance of the protein samples was measured at 590 nm. The experiment was done in triplicate to confirm the reproducibility.

2.11. Western Blotting. Briefly, cells were put in RIPA buffer, and protease inhibitors were added. All samples were subjected to sonication for 10 s to break the cell membranes. Centrifugation was done for the samples at 20,000g for 30 min at 4 °C. Protein concentration was determined using the Bradford reagent. SDS-PAGE was run to resolve proteins onto gels, which were then transferred to PVDF membranes. The blocking was done by dipping the PVDF membrane into 5% milk dissolved in PBST for 1 h at room temperature (RT). Then, the membrane was treated with primary antibodies overnight at 4 °C and then incubated with HRP-conjugated secondary antibodies for 1 h at RT. The Clarity ECL substrate was used to develop protein bands. Semiquantitative analysis was done using ImageJ software utilizing densitometry.

2.12. Statistical Analysis. Results were expressed as mean ± standard error of mean. All data were analyzed using analysis of variance (ANOVA) followed by Tukey’s test. Values of p < 0.05 were considered as significant. All the statistical analyses were performed using GraphPad Prism 5 software (Graph Pad Software, Inc., San Diego, CA).

3. RESULTS AND DISCUSSION

Colorectal cancer (CRC) is a global health concern, and targeted cancer therapy selective toward cancer cells/tissue may lead to reduced collateral damage to normal cells. Novel drug targets need to be explored for effective synthetic lethality-mediated cancer therapy. In this study, for the first time, we have established that peroxiredoxin-2 (PRDX2) has a synthetic lethal interaction with checkpoint kinase 2 (CHEK2) for the selective targeting of CHEK2-null colorectal cancer cells by inhibiting the PRDX2 enzyme using NCA, a PRDX2 inhibitor. Before executing the experiments to validate the synthetic lethal interaction between PRDX2 and CHEK2, we have performed western blotting experiments to confirm the presence/absence of CHEK2 in HCT116 and in CHEK2-null HCT116 cells (Figure S2). In addition to that, we have performed western blotting again to confirm the presence of PRDX2 protein expression both in HCT116 and in CHEK2-null HCT116 cells (Figure S2).

We have optimized the concentration of siRNA that exhibited the maximum silencing of PRDX2. We used various concentrations of siRNA for PRDX2 silencing in HCT116 cells and confirm the protein expression using western blotting. Our data showed that 75 nM siPRDX2 induced the maximum inhibition of the PRDX2 protein, as confirmed by western blotting, without inducing cytotoxicity at 75 nM concentration (Figure 1).

![Figure 1. Western blotting image for PRDX2 and GAPDH.](image)

We then performed siRNA-mediated silencing of PRDX2 in CHEK2-null HCT116 cells, and siGAPDH was used as a negative control. We performed high-content imaging using a Cytation-5 (CTYTSMV, BioTek Instruments Inc.) and a 4× objective was used to collect five nonoverlapping images. Our results showed that the numbers of DAPI-stained nuclei of CHEK2-null HCT116 cells markedly reduced after treatment with siPRDX2 as compared to siGAPDH as shown in Figure 2A. Furthermore, cell counts of CHEK2-null HCT116 cells decreased significantly after siRNA-mediated PRDX2 silencing as compared to siGAPDH. (Figure 2B), while there was no significant reduction in the HCT116 cell population after siPRDX2 treatment as compared to siGAPDH. These results suggest that there is a synthetic lethal interaction between PRDX2 and CHEK2.

N-carbamoyl alanine (NCA), a PRDX2 enzyme inhibitor, can be used as a drug candidate for the selective targeting of CHEK2-null HCT116 cells, while sparing HCT116 cells having a wild-type normal expression of CHEK2. MTT assay was performed to assess the dose–response curve (Figure 3) using different concentrations of NCA drug (ranging from 1
nM to 1 M with a 10-fold increase in the NCA dose) to determine the effective concentration at which 50% cells die (EC50). The calculated EC50 value for HCT116 cells was 5.56 μM, while the EC50 value for CHEK2-null HCT116 cells was 0.29 μM (Table 1). Interestingly, the EC50 dose for CHEK2-null HCT116 cells exerted minimal effects on HCT116 cells. Thus, in all subsequent experiments, the EC50 dose for CHEK2-null HCT116 cells was used.

After determining the EC50 value using the dose–response curve, we again treated CHEK2-null HCT116 cells with DMSO (control) and NCA (0.29 μM, EC50) and performed high-content imaging using a Cytation-5 similarly as aforementioned in siRNA-based experiments. Our data suggest that as compared to the DMSO control, NCA treatment leads to markedly reduced CHEK2-null HCT116 cells as shown by the DAPI-stained nuclear images (Figure 4A). In addition, the cell population of CHEK2-null HCT116 cells reduced significantly (p < 0.001) after NCA treatment as compared to the DMSO control (Figure 4B). These findings suggest that PRDX2 and CHEK2 have a strong synthetic lethal interaction that can be exploited for the specific targeting of CRC cells having CHEK2 defects.

In the detoxification pathway, PRDX2 is responsible for the conversion of H2O2 to H2O and oxygen. The level of intracellular ROS (H2O2) increased due to inhibition of the PRDX2 enzyme, and H2O2 is well-reported to induce DNA damage (DNA DSBs).23,24 CHEK2 plays an important role in the repair of cellular DNA DSBs through the homologous recombination repair (HRR) mechanism. Defects in CHEK2 may lead to an inadequate function of the HRR pathway that is crucial for the repair of DNA DSBs. Thus, inhibition of the PRDX2 enzyme using NCA leads to an enhanced level of ROS, which induces DNA DSBs, and wild-type functional CHEK2 is needed to repair ROS-induced DNA DSBs. Therefore, defects in CHEK2 may result in persistent DNA DSBs within the cells, and to maintain its integrity, cells are expected to adopt an alternative nonhomologous end joining (NHEJ) DNA repair mechanism, also known as an error-prone mechanism. The NHEJ pathway results in cataclysmic genomic changes due to the accumulation of errors and ultimately leads to cell death due to excessive accretion of intracellular cataclysmic genomic modifications.

Figure 2. (A) Images of DAPI-stained nuclei of CHEK2-null HCT116 cells after treatment with siRNA for GAPDH (negative control) and PRDX2. (Objective: 4×). Scale bar: 1000 μm. (B) Bar graph shows that treatment with siPRDX2 leads to significantly (p < 0.001) reduced cell numbers of CHEK2-null HCT116 cells as compared to HCT116 cells, while there was no significant difference in cell numbers between CHEK2-null HCT116 cells and HCT116 cells after treatment with siGAPDH. GAPDH was the negative control.

Figure 3. Graph illustrate the % cell viability of HCT116 cells and CHEK2-null HCT116 cells after treatment with different concentrations of NCA to generate a dose–response curve.

Figure 4. (A) Images of DAPI-stained nuclei of CHEK2-null HCT116 cells after treatment with DMSO and NCA. (Objective: 4×). Scale bar: 1000 μm. (B) Bar graph shows treatment with NCA leads to significantly (p < 0.001) reduced cell numbers of CHEK2-null HCT116 cells as compared to the DMSO control.
Based on the abovementioned facts, to further understand the mechanism of selective cell death of CHEK2-null HCT116 cells, we performed DCFHDA assay for the estimation of total reactive oxygen species (ROS). We found that NCA treatment results in an increased level of total intracellular ROS in both HCT116 cells and CHEK2-null HCT116 cells as compared to the DMSO control as shown by the green fluorescence (Figure 5).

The inhibition of the PRDX2 enzyme particularly increased the level of H$_2$O$_2$ as PRDX2 is responsible for converting H$_2$O$_2$ to H$_2$O and oxygen. We performed the Amplex Red assay to estimate the level of H$_2$O$_2$ generated in both HCT116 cells and CHEK2-null HCT116 cells. We found that both cell types generated an increased level of H$_2$O$_2$ after treatment with NCA as compared to the DMSO control (Figure S3).

The increased level of H$_2$O$_2$ in cells is reported to induce DNA damage (DSBs); therefore, to further investigate the deleterious effects of H$_2$O$_2$ on DNA, we performed the immunofluorescence assay to study the biomarkers of DNA DSBs such as 53BP1 and γ-H2AX. The p53-binding protein 1 (53BP1) is a protein, which binds to the DNA binding domain (DBD) of p53. In response to DNA DSBs, 53BP1 translocate to the DSB site and forms multiple nuclear foci. Furthermore, 53BP1 recruits other proteins involved in signaling cascades and the repair of DNA DSBs. Importantly, 53BP1 favors the NHEJ DNA repair pathway and prevents the HRR pathway.\textsuperscript{28,29} Similarly, in response to DNA DSBs, the histone variant H$_2$AX is rapidly phosphorylated to γ-H2AX, which is a hallmark of early cell response to DSBs.\textsuperscript{30} Our results showed that treatment with NCA resulted in an increased expression of both the biomarkers of DNA DSBs such as 53BP1 and γ-H2AX, which is a hallmark of early cell response to DSBs.\textsuperscript{30} Our results showed that treatment with NCA resulted in an increased expression of both the biomarkers of DNA DSBs such as 53BP1 and γ-H2AX, particularly in CHEK2-null HCT116 cells as compared to HCT116 cells, while DMSO (vehicle control) did not enhance the expression of 53BP1 and γ-H2AX in both cell types (Figures 6 and 7).

Irreparable DNA damage in cells fosters the orchestration of different caspases, which leads to apoptosis. Cleaved caspase-3 is a main executioner caspase, and its expression or activity markedly enhanced in cells that are programmed to undergo apoptotic cell death.\textsuperscript{31} Our results further showed that
CHEK2-Wild type CRC Cells

Control (DMSO)

NCA (0.28 μM)

DAPI Cleaved Caspase3 Merge

Figure 8. Representative images show that treatment with NCA results in an increased expression of cleaved caspase-3 in CHEK2-null HCT116 cells as compared to HCT116 cells. DMSO was used as a negative control.

treatment with NCA resulted in an increased expression of cleaved caspase-3 in CHEK2-null HCT116 cells as compared to HCT116 cells, while DMSO (vehicle control) did not enhance the expression of cleaved caspase-3 in both cell types (HCT116 cells and CHEK2-null HCT116 cells) (Figure 8).

4. CONCLUSIONS

Our cell-based studies strongly suggest that there is a synthetic lethal interaction between PRDX2 and CHEK2, which could be exploited for the selective targeting of cancer cells having CHEK2 defects. This synthetic lethal interaction between PRDX2 and CHEK2 has wider applicability for selective cancer targeting as a CHEK2 defect has been reported to be present in various other cancer types.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c00527.

Details for the mutational status of the CHEK2 gene from online data on cBioportal for cancer genomics, western immunoblot analysis to validate CHEK2-null HCT116 cells for CHEK2 proficiency and deficiency, and level of H2O2 generated by the HCT116 cells and CHEK2-null HCT116 cells in the absence and presence of n-carbamoyl alanine (NCA) treatment (PDF)

## AUTHOR INFORMATION

### Corresponding Authors

Syed Shadab Raza — Laboratory for Stem Cell & Restorative Neurology, Department of Biotechnology, Era’s Lucknow Medical College Hospital, Lucknow 226003 Uttar Pradesh, India; Email: drsyedshadabenza@gmail.com

Rehan Khan — Chemical Biology Unit, Institute of Nano Science and Technology, Mohali 140306 Punjab, India; orcid.org/0000-0002-4599-5924; Phone: +91-172-2210075; Email: rehankhan@inst.ac.in

### Authors

Anas Ahmad — Chemical Biology Unit, Institute of Nano Science and Technology, Mohali 140306 Punjab, India; orcid.org/0000-0002-2406-1129; Phone: +91-172-2210075; Email: rehankhan@inst.ac.in

Ravi Prakash — Laboratory for Stem Cell & Restorative Neurology, Department of Biotechnology, Era’s Lucknow Medical College Hospital, Lucknow 226003 Uttar Pradesh, India

Mohd Shahnawaz Khan — Department of Biochemistry, College of Sciences, King Saud University, Riyadh 11451, Saudi Arabia; orcid.org/0000-0002-4599-5924

Nojood Altwaijry — Department of Biochemistry, College of Sciences, King Saud University, Riyadh 11451, Saudi Arabia

Muhammad Nadeem Asghar — Department of Medical Biology, University of Québec at Trois-Rivières, Trois-Rivières, Québec G9A SH7, Canada

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.2c00527

Notes

The authors declare no competing financial interest.

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