Open Access Original Article

Cisplatin and Oncolytic Adenoviral Vector Co-Treatment Induces Synergistic Proliferation Inhibitory Effects in Breast Cancer Cells

***Muhammad Tahir*[1]**
***Abdul Rehman*[2]**
***Adeel Riaz*[3]**

[1]College of Life Science and Bioengineering, Beijing University of Technology, Beijing 100124, P.R China.
[2,3]Department of Biochemistry, PMAS Arid Agriculture University, Rawalpindi 46000, Pakistan.

Address for Correspondence
Dr. Muhammad Tahir
College of Life Science and Bioengineering, Beijing University of Technology, Beijing 100124, P.R China
m.tahir.qau@hotmail.com

Abstract

Objective: To investigate the proliferation inhibitory effects of Cisplatin, CRAd mono, or Cis-CRAd combined therapy in MCF-10 breast cancer cells.

Methodology: In this prospective cohort study tumor inhibition activity of cisplatin, CRAd alone, and in combination, was studied. Breast cancer cells MCF-10 (ATCC, CRL-10317) were obtained from ATCC. These were cultured and propagated following the standard procedures.Cell viability assay and flow cytometry-based assay were used to measure the tumor cell growth inhibition, apoptosis induction, and cell cycle arresting ability of both anti-cancer agents in breast tumor cells. Further, the mRNA expression of p53 and its downstream target genes responsible for apoptosis induction and cell cycle arrest measured by RT-qPCR.

Results: Combined therapy with Cisplatin-CRAd significantly inhibited the cell growth, induced apoptosis, and blocked G2-M phase cell cycle transition in breast cancer cells. The mRNA expression analysis of p53 and its downstream genes responsible for apoptosis induction and cell cycle arrest was found out to be elevated in treated breast tumor cells.

Conclusion: Cisplatin, if used in combination with other competent and non-toxic anti-cancer agents like CRAd, can produce better anti-cancer effects at low doses. The results of this study indicate such a combined treatment approach may be investigated in animal models then at the clinical level.

Keywords: Adenoviral Vectors, Apoptosis, Cisplatin, Breast Cancer, p53, Cell cycle.

Introduction

Breast cancer is the most commonly diagnosed cancer and responsible for cancer- related deaths in women worldwide. Cisplatin has long being used for the treatment of solid tumors, including breast cancer. Although cisplatin proved to be a potent chemotherapeutic agent against cancer but associated with certain adverse effects, increase the dosage, and development of drug resistance. Combined therapy with cisplatin and other anti-cancer agents may help in coping limitations associated with cisplatin monotherapy. Adenoviral vectors have shown potent anti-tumor activity in many preclinical and clinical studies. Adenoviral vectors offer an effective and non-toxic approach for the treatment of cancer. In present study, the conditionally replicating oncolytic adenoviral vector (CRAd) with cisplatin, were combined, to achieve better proliferation inhibitory effects in breast cancer cells.

Breast cancer is the most frequent neoplasm in women and is hard to treat once metastasize to other parts of the body.[1] Currently, breast cancer is managed by radiotherapy, surgery, chemotherapy, or a combination of these treatment approaches. Chemotherapy remained the frontline strategy to treat solid tumors, including breast cancer. Cisplatin (Cis), a platinum-based chemotherapeutic agent, is being utilized to combat breast cancer for decades. Cisplatin has produced promising therapeutic effects in many cancer types, including testicular, lung, breast, head and neck cancer, cervical and bladder cancer.[2, 3] Cisplatin mainly

Authorship Contribution: [1]Designing and conducting experiments in this study.[2]Experimentations, [3]Data analysis and manuscript writing

Funding Source: Key Programs of Beijing Municipal Science and Technology Commission
Grant # K2015311201501
Conflict of Interest: none

Received: Nov 21, 2020
Accepted: Jan 18, 2021

acts by damaging DNA, by producing stress signals in the cell. These stress signal activates a cascade of events which lead to the inhibition of replication, transcription, and translation processes, and ultimately cancer cell is eliminated.[4-7]

Despite offering meaningful anti-cancer effects, long-term use and high doses of cisplatin are associated with the development of drug resistance and adverse effects, including myelosuppression, gastrointestinal disorder, cardiopathy and renal toxicities in cancer patients.[8, 9] Therefore, to benefit from the classical anti-cancer effects of cisplatin, there is a need to develop new combined treatment strategies using low doses of chemotherapeutic agents like cisplatin and other potent, less toxic anti-cancer agents.

Virotherapy is considered a favourable option to be used in combined therapy with chemotherapy agents. Viral vectors have shown the tendencies to produce synergistic anticancer effects in combination therapies, especially in chemo-virotherapy.[10] Oncolytic adenoviruses (OAds) have been extensively investigated in many studies. OAds offer multiple advantages like high transduction efficiency, cost effective production, can be produced conveniently at large scale. Additionally, these could suppress the tumor by supplying genes of interest (tumor suppressor genes) to tumor site as well as performing their default oncolytic activity in tumor cells.[11]

Conditionally replication-competent adenoviruses (CRAds) tend to replicate in and lyse the tumor cells selectively. These are constructed by modifying viruses in such a way that these become tumor-selective, but their replication ability stays intact. For such purpose, the adenoviral replication driving E1A/E1B genes are put under the control of specific promoters, which are highly expressed in tumor cells but not in healthy body cells. One such type of promoter is the survivin promoter. Survivin is a member of the inhibitor of the apoptosis protein family (IAP). Many studies have demonstrated transcriptional upregulation of surviving promoter in tumors.[12, 13] Survivin promoter governed replication could enable OAdvs (CRAds) to replicate and follow their lytic cycle only in tumor cells; thus, tumor cells will be lysed, and normal cells will be spared.

Methodology

Cisplatin was purchased from MedChem Express (Shanghai, China). Survivin promoter regulated conditionally replicating adenovirus (CRAd) was developed in our research laboratory as described previously.[11] Ad-Luc (empty vector) was used as a control.

Cell Viability assay: MCF-10 breast cancer cells were cultured in 24 well plates containing 10% FBS added DMEM for 24h (37°C, 5%CO2). Cultured cells were transferred to 96 well plates (3x 103 cells/ well) containing 2% FBS added DMEM. Breast cancer cells were treated with cisplatin and CRAd, at different concentrations for 48h (37°C, 5%CO2). After incubation, 20 µl of MTT (5mg/ml) reagent were added to each well. After 4 h culture medium in wells was discarded and 200 µl of DMSO was added, and 96 well plates were incubated for 10 min at room temperature, then cell viability was measured by taking OD of each well at 490nm using a microplate reader and expressed as a percentage of control cell viability.

Combination studies: MCF-10 breast cancer cells were treated with different concentrations of cisplatin (1, 4, 16, 64) and a fixed concentration of CRAd (4 MOI). Cell viability assay data noted, and data were subjected to CI-isobologram method to evaluate the possible synergistic anti-cancer effects of Cis-CRAd combined therapy using CompuSyn 2.0 program created by Chou and Martin.[14] Combination index (CI) < 1, CI=1, CI > 1 represent synergistic effects, additive effects and antagonistic effects, respectively.

Apoptosis analysis: Breast cancer cells MCF-10 were cultured in 6–well plates for 24h, then treated with Cis, CRAd, or combination of Cis and CRAd. 48h after treatment cells were isolated and double-stained with Annexin V-FITC/ Propidium iodide (Invitrogen, Carlsbad, CA, USA). Staining was performed as per the standard protocol. Stained cells were analyzed for the onset of apoptosis through flow cytometry using FACScalibur™

Cell cycle analysis: MCF-10 cells were treated with Cis, CRAd, or combination of Cis and CRAd for 48 h. At the end of treatment, cells were isolated and fixed with 70% ice-cold ethanol. Cells were incubated at - 20 °C for 48 h. After incubation, cells were washed with PBS thrice. Following washing 50 µg/mL of Propidium Iodide (PI)

and 25 µg/mL of RNase A (Invitrogen, Carlsbad, CA, USA) were added, and cells were kept at room temperature (in the dark) for 15min. These stained cells were analyzed for changes in cell cycle using FACScalibur™ (Becton Dickinson) flow cytometer. FlowJo_V10 software used to analyze the data.

RNA isolation and RT-qPCR: MCF-10 cells were cultured for 24 h then treated with anti-cancer agents for 48h. After treatment cells were collected and Total RNA was isolated using TRIzol reagent (Beyotime, Haimen, China) as per standard protocol. The RNA concentration was quantified using Nano Drop 2000 (Thermo Fisher Scientific, Inc.). To measure the expression, RNA was reverse transcribed through RT-PCR to obtain cDNA. To quantify the level of mRNA expression of p53, 14-3-3-σ, CDC25, Cdc2, Bax, Bcl2, caspase 3 and caspase 9, the SYBR Premix Ex TaqTM kit was utilized. GAPDH was used as an internal control. Primers used in this study are listed in Table I. Experiments performed thrice independently. The relative expression of genes was calculated using the $2^{-\Delta\Delta Cq}$ method.[15]

The data sets from three independent experiments were subjected to statistical analysis (Mean ± Standard deviation, one way ANOVA and post hoc analysis) to evaluate the significance of experimental results. For statistical analysis, OriginPro 9 software was used. Results showed *p-value < 0.05 were considered significant.

Results

Cisplatin, CRAd mono or combined treatment inhibited the growth of breast cancer cells successfully .Cytotoxicity of Cisplatin, CRAd, or combination of Cis and CRAd in MCF-10 breast cancer cells evaluated. MCF-10 cells treated with CRAd (1, 4, 16, 64 MOI) and Cisplatin (1, 4, 16, 64 µg/ml) for 48h, then cytotoxic effects of treatments evaluated through MTT or cell viability assay. Both anti-cancer agents inhibited the growth of breast cancer cells in a dose-dependent manner (Figure 1 A & B). Half, maximal inhibitory concentration (IC50) values of Cisplatin and CRAd, were observed 16 µg/ml and 16 MOI, respectively.

CRAd at 4 MOI (< IC50 Value) concentration was combined with 1, 4, 16, 64 µg/ml cisplatin concentrations. Combined treatment with Cisplatin and CRAds produced substantial proliferation inhibitory effects compared to Cis or CRAd alone treatments. Combined treatment remarkably reduced the IC50 value compared to Cis or CRAd alone treatment (CRAd 4 vs. 16 MOI, Cisplatin 4 vs. 16 µg/ml). (Figure 1 C). Findings of cell viability assay revealed combined treatment significantly (*p< 0.05) inhibit the cancer cell growth compared to the alone treatments.

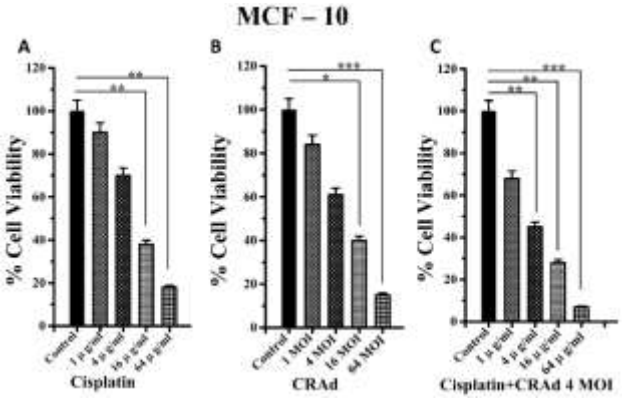


Figure 1:Cisplatin, CRAd mono or combined treatment in Breast cancer cells.

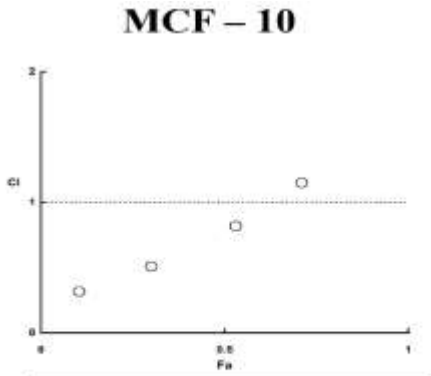
Synergy analysis was performed on cell viability assay data to evaluate the possible synergistic effects of Cis-

Table I: Primers for RT-qPCR

Genes	Primers		Ref.
	Sense (5'—3')	Antisense (5'—3')	
p53	ATGTTTTGCCAACTGGCCAAG	TGAGCAGCGCTCATGGTG	(16)
14-3-3-σ	ACTACGAGATCGCCAACAGC	CAGTGTCAGGTTGTCTCGCA	(17)
CDC25C	AGTCAGAAGGAACTGCATGAG	CAGAGAACGGCACATTCGAG	(18)
Cdc2	ACAGGTCAAGTGGTAGCCATGA	CCTGGAATCCTGCATAAGCA	(19)
Bax	ATGTTTTCTGACGGCAACTTC	AGTCCAATGTCCAGCCCAT	(16)
Bcl-2	ATGTGTGTGGAGACCGTCAA	GCCGTACAGTTCCACAAAGG	(16)
Caspase-9	CATTTCATGGTGGAGGTGAAG	GGGAACTGCAGGTGGCTG	(16)
Caspase-3	TGTTTGTGTGCTTCTGAGCC	CACGCCATGTCATCATCAAC	(16)
GAPDH	GCAAATTCCATGGCACCGT	TCGCCCCACTTGATTTTGG	(19)

CRAd combined therapy. Combined treatment with CRAd 4 MOI (Fixed concentration) and Cisplatin 1, 4, 16, 64 µg/ml (Variable concentrations) in MCF-10 exhibited fraction-affected (Fa) values 0.71, 0.53, 0.31, 0.10 and Combination index (CI) values 1.15, 0.82, 0.51, 0.28. Data indicate that tumor inhibitory effects of combined treatment in breast cancer cells are highly synergistic (CI< 1). The most effective synergistic effects observed in combined treatment with CRAd 4 MOI and Cisplatin 4 µg/ml concentrations (Figure 2)

MCF-10 breast cancer cells treated with (A) Cisplatin, (B) CRAdor (C) a combination of Cisplatin and for 48 h. The cytotoxic effects of the anti-cancer agents assessed through the MTT assay. Results shown as means ± standard deviation of three independent experiments. *p<0.05, **p<0.01 and ***p<0.005 vs. control cells. Measurement of the Combination Index (CI) of Cisplatin and CRAd in MCF-10 cells were carried out using the CI-isobologram method. CI < 1 represents the synergistic effects. Cisplatin, CRAd mono or combined treatment achieved apoptosis through P53 dependent intrinsic apoptotic patway in breast cancer cells



Group	CRAd (MOI)	Cisplatin (µg/ml)	Fa	CI
1	4	1	0.71	1.15
2	4	4	0.53	0.82
3	4	16	0.31	0.51
4	4	64	0.10	0.28

Figure 2. Synergistic effects of Cisplatin and CRAd combined treatment.

Cisplatin and CRAd, either alone or in combination, induced apoptosis compared to control cells. The rate of apoptosis was significant (*p<0.05) in Cis-CRAd co-treated cells (83%) compared to Cisplatin (30%) and CRAd (38%) mono treated cells (Fig.3 A&B). The apoptosis induction ability of both anti-cancer agents was further affirmed by measuring the level of mRNA expression of genes involved in the apoptotic pathway. mRNA expression analysis revealed the higher mRNA expression of pro-apoptotic genes Bax, Caspase 9, Caspase 3, and lower expression of anti-apoptotic gene Bcl2 in Cisplatin and CRAd mono or combined treated cells compared to control cells. Co-treatment greatly enhanced the expression compared to mono treatments. This mRNA expression pattern indicates that treatment approaches employed in this study induced apoptosis in MCF-10 breast cancer cells through initiating intrinsic apoptotic pathway (Fig 3C).

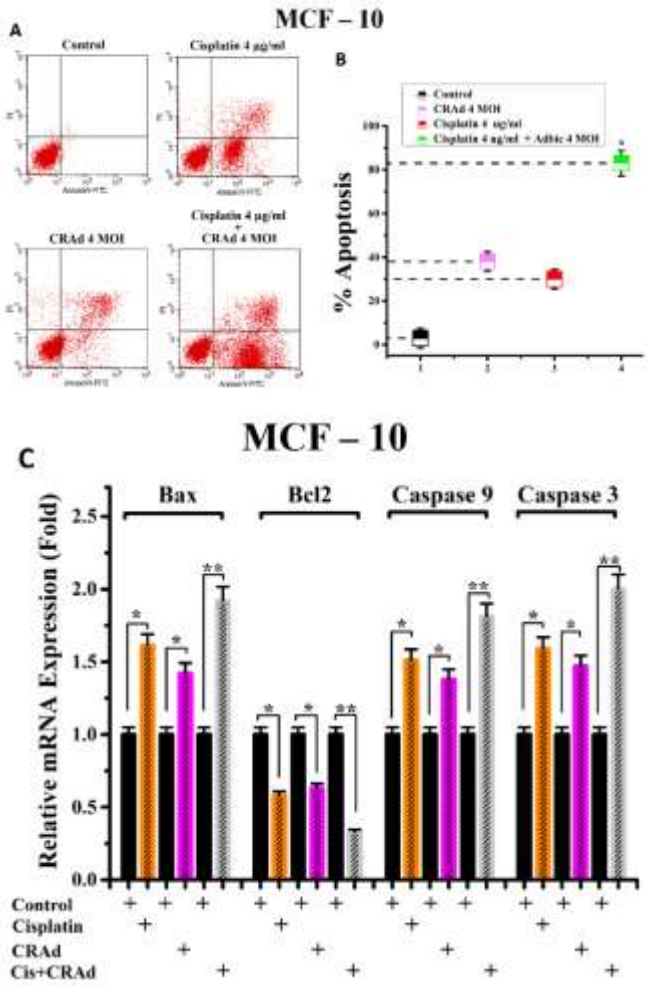


Figure 3. Apoptosis induction analysis. (A)

MCF-10 cells were treated with Cisplatin, CRAd alone or Cis-CRAd combination for 48 h, and cells were double-stained with Annexin V-FITC/PI staining and subjected to flow cytometry analysis by FASCcalibur. (B) The histogram represents the apoptosis rate in breast cancer cells. Data are representative of three independent experiments with mean ± SD. *p<0.05 vs. control cells.(C) mRNA Expression analysis of genes involved in

apoptosis: mRNA expression of Bax, Bcl2, Caspase 9 and Caspase 3 proteins in breast cancer cells after 48h treatment with Cisplatin, CRAd alone or Cis-CRAd combination. The expression of genes of apoptotic pathway was normalized to that of GAPDH. Data are representative of three independent experiments with mean ± SD. *p<0.05, **p<0.01 vs. control cells.

MCF-10 breast cancer cells treated with CRAds (4 MOI), Cisplatin (4 µg/ml), or combination of both for 48h. The populations of treated cells in different phases of the cell cycle were determined by staining the cells with PI stain followed by flow cytometry. Cell cycle analysis revealed a higher distribution of Cisplatin, CRAds mono, or combined treated cells in the G2/M phase compared to control cells (Fig. 4 A). Results indicate that treatment with anti-cancer agents either alone or combined blocked the G2-M phase transition of the cell cycle, but the proportion of cells that received combined treatment was significantly (*p< 0.05) higher in the G2/M phase compared to cells received mono treatments (Fig. 4 B).

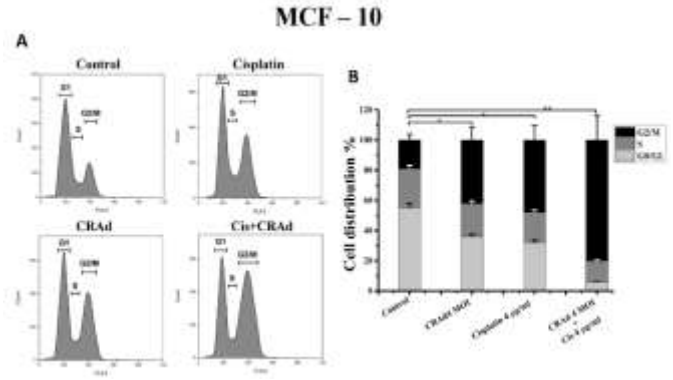


Figure 4. Effects of Cisplatin, CRAd alone, or Cis-CRAd combined treatment on breast cancer cell cycle. (A)

MCF-10 cells were subjected to cell cycle analysis after 48h treatment with both anti-cancer agents. The distribution of treated cells in different phases of the cell cycle was analyzed through flow cytometry by FASCcalibur.(B) The histogram represents the apoptosis rate in breast cancer cells. Data are representative of three independent experiments with mean ± SD shown. *p<0.05, **p<0.01 vs. control cells.

Cisplatin, CRAds mono or combined treatment achieved P53 pathway lead G2-M cell cycle arrest. mRNA expression analysis revealed the high mRNA expression of p53 and 14-3-3-σ genes while low expression of CDC25 and Cdc2 genes. This expression pattern suggests that CRAds, Cisplatin treatment upregulated p53 which performed its tumor suppressor role and arrested the cell cycle.

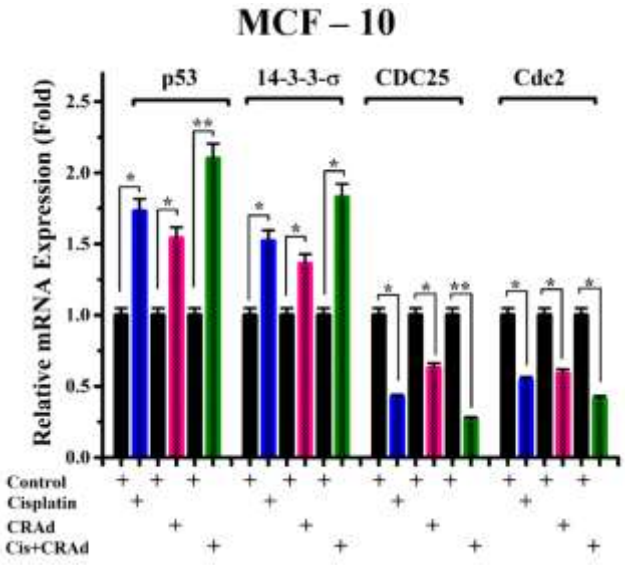


Figure 5. Expression analysis of cell cycle regulatory genes.

mRNA expression of p53, 14-3-3-σ, CDC25C and Cdc2 in breast cancer cells after 48h treatment with Cisplatin, CRAd alone or Cis-CRAd combination. The expression of genes involved in cell cycle was normalized to that of GAPDH. Data are representative of three independent experiments with mean ± SD. *p<0.05, **p<0.01 vs. control cells.

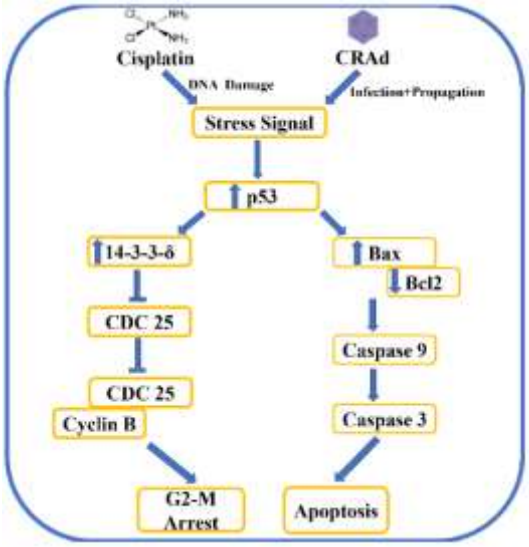


Figure 6. Proposed molecular mechanism of Cis-CRAd treatment induced breast cancer cell proliferation inhibition.

Discussion

Despite recent advances in cancer treatment strategies, there is still a need for devising and investigating new anti-cancer approaches to achieve better treatment outcomes. Chemotherapy associated adverse effects and drug resistance issues significantly undermine the potent therapeutic impact and contribute to minimizing the survival rate and worsening the life quality in cancer patients. Combining chemotherapy agents with less toxic agents could help in overcoming the existing drugs-related issues in cancer treatment.

Oncolytic adenoviruses (OAds), which kill tumor cells by oncolysis and host immune activation, are gaining popularity in cancer therapy research. Their genomic structure is also well known, which offers the possibility of engineering the viral vectors capable of selectively killing cancer cells without harming healthy cells.[20, 21] Presently, several OAds based anti-cancer treatment strategies are being investigated in different phases (I – III) of clinical trials.[20] Cisplatin is a potent chemotherapy agent that kills the tumor cells by breaking the DNA strands and renders the cells unable to replicate. However, over time it is observed that at a certain point, cisplatin receiving patients suffer from adverse effects and develop cisplatin resistance. Long term use of high doses of cisplatin might contribute to developing drug resistance and side effects.

In our current study, we proposed the hypothesis that the combination of cisplatin with CRAds could produce synergistic anti-cancer effects at a low dosage than the dosage used in monotherapy with these agents. To investigate this notion, we treated breast cancer cells with cisplatin, CRAd alone, or in a combination of both agents. Cisplatin is known to interfere with the DNA and inhibits the proliferation of tumor cells.[22] Oncolytic adenoviral vectors kill the cells through oncolysis.[23] In this study, findings of cell viability assay showed that cisplatin and CRAd mono and combined treatments induced cytotoxic effects in breast cancer cells. It is also observed that cisplatin and CRAd co-treatment produced synergistic toxic effects. This suggests that both anti-cancer agents augmented each other's cytotoxic activity and achieved significant (*$p<0.05$) tumor cell inhibition at low concentrations compared to mono-treatments. In response to the cytotoxic actions of cisplatin, cells produce stress signals. Similarly, entry and propagation of oncolytic adenoviral vectors in the cells also drive the cell to generate stress signals. These stress signals contribute to activating various intrinsic molecular pathways, which then initiate programmed cell death, cell cycle arrest or other growth inhibitory mechanisms to eliminate the affected cells.

P53 is one of the most important tumor suppressor proteins, which is activated in response to stress signals. Following activation, p53 triggered the transcriptional activation of its target genes, which then perform their default activities to halt the growth of cell. It is well established that P53 pathway could trigger intrinsic and extrinsic apoptotic pathways, and cell cycle arrest at G1-S or G2-M phase.[24] P53 can initiate an intrinsic apoptotic pathway by activating Bax and blocking the activity of Bcl2. Bax then triggers caspase 9 and caspase 3, which ultimately accomplish apoptosis. P53 is also well capable of arresting the cell cycle at G2-M phase. Activated P53 upregulates the expression of 14-3-3δ, which is a cell cycle regulatory protein. 14-3-3δ then binds to CDC25C and blocks its activity. CDC25 is a phosphatase, which promote the formation of Cyclin B/Cdc2 complex. Cyclin B/Cdc2 is a kinase complex, which has essential role in G2-M phase transition of cell cycle. Unavailability of Cyclin B/Cdc2 complex results in cell cycle arrest at G2/M phase.[25,26] In our study, Cisplatin and CRAd mono or co-treatment instigated apoptosis and arrested cell cycle at G2/M phase. Co-treatment elicited much enhanced growth inhibitory effects. To explore the possible molecular mechanisms attributed to inducing apoptosis and cell cycle arrest, we evaluated the mRNA expression of genes of P53 pathway responsible for inducing apoptosis and cell cycle arrest. Results indicated high level of expression of p53 and its associated pro-apoptotic Bax, Caspase 9, Caspase 3 genes and low expression of anti-apoptotic Bcl2 gene in Cisplatin and CRAd treated breast cancer cells. Likewise, mRNA expression analysis of cell cycle regulatory genes showed elevated level of expression of 14-3-3-σ gene and downregulation of CDC25C and Cdc2 genes in treated cells. This mRNA expression pattern clearly indicates Cisplatin, CRAd alone or combined treatment upregulated the P53 level and activated the P53 pathway. The P53 pathway then executed its classical tumor cell growth inhibitory functions by stimulating intrinsic apoptosis pathway and arresting cell cycle at G2-M phase.

Conclusion

1. Cis-CRAd co-treatment synergistically inhibited tumor cell growth compared to mono-treatments. Cis-CRAd treatment achieved significant inhibitory effects at low doses than doses utilized in mono-treatments. 2.Treatment options employed in this study arrested the cell cycle at the G2/M phase and induced apoptosis, possibly by activating the p53 pathway.
3. Molecular mechanism underlying achieving proliferation inhibitory effects in treated breast cancer cells might be the upregulation of P53, which in association with its target genes accomplished proliferation inhibitory effects.
4. Cis-CRAd co-treatment may provide a possibility of devising a new approach for breast cancer treatment.

Acknowledgements: We are very thankful to Ming Zhao in Torrey Pines Institute for Molecular Studies for her technical assistance, and Beijing Inmay Future Biopharma Inc. to provide experimental materials.

References

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA: a cancer journal for clinicians. 2015;65(2):87-108.
2. Ho GY, Woodward N, Coward JI. Cisplatin versus carboplatin: comparative review of therapeutic management in solid malignancies. Critical Reviews in Oncology/Hematology. 2016;102:37-46.
3. Yang Y, Adebali O, Wu G, Selby CP, Chiou Y-Y. Cisplatin-DNA adduct repair of transcribed genes is controlled by two circadian programs in mouse tissues. Proceedings of the National Academy of Sciences. 2018;115(21):E4777-E85.
4. Al-Lazikani B, Banerji U, Workman P. Combinatorial drug therapy for cancer in the post-genomic era. Nature Biotechnology. 2012;30(7):679.
5. Amable L. Cisplatin resistance and opportunities for precision medicine. Pharmacological Research. 2016;106:27-36.
6. Garraway LA, Jänne PA. Circumventing cancer drug resistance in the era of personalized medicine. Cancer Discovery. 2012;2(3):214-26.
7. Novello S, Barlesi F, Califano R, Cufer T, Ekman S. Metastatic non-small-cell lung cancer: ESMO Clinical Practice Guidelines. Annals of Oncology. 2016;27(suppl_5):v1-v27.
8. Gottesman MM, Pastan I. Biochemistry of multidrug resistance mediated by the multidrug transporter. Annual Review of Bbiochemistry. 1993;62(1):385-427.
9. Sharom FJ. ABC multidrug transporters: structure, function and role in chemoresistance. 2008.
10. Muhammad T, Sakhawat A, Khan AA, Huang H, Khan HRl. Aloperine in combination with therapeutic adenoviral vector synergistically suppressed the growth of non-small cell lung cancer. J Cancer Res Clin Oncol . 2020;146(4):861-74.
11. Muhammad T, Sakhawat A, Khan AA, Ma L, Gjerset RA, Huang Y. Mesenchymal stem cell-mediated delivery of therapeutic adenoviral vectors to prostate cancer. Stem Cell Res. Ther. 2019;10(1):190.
12. Bao R, Connolly DC, Murphy M, Green J, Weinstein JKl. Activation of cancer-specific gene expression by the survivin promoter. J Natl Cancer Inst. 2002;94(7):522-28.
13. Satoh K, Kaneko K, Hirota M, Masamune A. Expression of survivin is correlated with cancer cell apoptosis and is involved in the development of human pancreatic duct cell tumors. International Journal of American Cancer Society. 2001;92(2):271-78.
14. Chou T-C. Drug combination studies and their synergy quantification using the Chou-Talalay method. Cancer Research. 2010;70(2):440-6.
15. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. Methods. 2001;25(4):402-8.
16. Mitupatum T, Aree K, Kittisenachai S, Roytrakul S. mRNA expression of Bax, Bcl-2, p53, cathepsin B, caspase-3 and caspase-9 in the HepG2 cell line following induction by a novel monoclonal Ab Hep88 mAb. Asian Pac J Cancer Prev. 2016;17(2):703-12.
17. Maeda R, Tamashiro H, Takano K, Takahashi Hl. TBP-like protein (TLP) disrupts the p53-MDM2 interaction and induces long-lasting p53 activation. Journal of Biological Chemistry. 2017;292(8):3201-12.
18. Tane S, Kubota M, Okayama H, Ikenishi A. Repression of cyclin D1 expression is necessary for the maintenance of cell cycle exit in adult mammalian cardiomyocytes. J. Biol. Chem. 2014;289(26):18033-44.
19. Amatori S, Persico G, Fanelli M. Real-time quantitative PCR array to study drug-induced changes of gene expression in tumor cell lines. J Cancer Metastasis Treat. 2017;3:90-9.
20. Lee JM, Dedhar S, Kalluri R, Thompson EW. The epithelial–mesenchymal transition: new insights in signaling, development, and disease. The Journal of cell biology. 2006;172(7):973-81.
21. Yuan CH, Horng CT, Lee CF, Chiang NN, Tsai FJ. Epigallocatechin gallate sensitizes cisplatin-resistant oral cancer CAR cell apoptosis and autophagy through stimulating AKT/STAT3 pathway and suppressing multidrug resistance 1 signaling. Environmental toxicology. 2017;32(3):845-55.
22. Ricci MS, Zong W-X. Chemotherapeutic approaches for targeting cell death pathways. The oncologist. 2006;11(4):342.
23. Eager R, Nemunaitis J. Clinical development directions in oncolytic viral therapy. Cancer gene therapy. 2011;18(5):305-17.
24. Harris SL, Levine AJ. The p53 pathway: positive and negative feedback loops. Oncogene. 2005;24(17):2899-908.
25. Iliakis G, Wang Y, Guan J, Wang H. DNA damage checkpoint control in cells exposed to ionizing radiation. Oncogene. 2003;22(37):5834-47.
26. Lane DP, Cheok CF, Lain S. p53-based cancer therapy. Cold Spring Harbor perspectives in biology. 2010;2(9):a001222.