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AdenoCure 3X: Establishing a Sensitized Cancer Cell Model to Accelerate Novel Drug Discovery

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Abstract: Cancer is a major cause mortality globally, and chemotherapy is frequently employed as a treatment for cancer. Regrettably, cancers possess the ability for developing resistance to conventional treatments, and the rising recurrence of these drug-resistant cancers requires further research and the advancement of treatment approaches. To address the growing challenge of chemoresistance, this study presents a novel approach for developing a sensitized cancer cell model using AdenoCure 3X to accelerate the discovery of novel drugs. We constructed a tetracistronic adenoviral vector, Ad-MBRG for targeted delivery of tumour suppressor genes MOAP-1, Bax, and RASSF1A, known to be epigenetically silenced in many cancers and contribute to apoptotic pathway dysfunction. This approach aims to restore apoptotic signaling and enhance cancer cell sensitivity to treatment. The study outlines a multi-step methodology for establishing the Ad-MBRG-mediated sensitized cell model, including amplification and quantification of Ad-MBRG particles, optimization of infection efficiency in the MCF7-CR cancer cell line, and evaluation of their sensitivity compared to non-infected controls. Furthermore, we explored the potential synergistic effects between Ad-MBRG infection and anti-cancer compounds derived from natural products. This model holds promise for screening anti-cancer compounds, particularly those from natural sources, and evaluating their potential to overcome chemoresistance. This paves the way for further exploration of adenovirus-mediated gene therapy as a platform for novel drug discovery and improved cancer treatment strategies.

Keywords: Adenovirus; Chemo-sensitization; Cancer Cell Model; Cancer Gene Therapy

1. Introduction

Cancer is a major cause mortality globally, and chemotherapy is frequently employed as a treatment for cancer, often leading to severe side effects associated with the cytotoxicity caused by the dosage[1,2]. Regrettably, cancers possess the ability for developing resistance to conventional treatments, and the rising recurrence of these drug-resistant cancers requires further research and the advancement of treatment approaches.

Chemo-resistance arises from various mechanisms, such as the activation of DNA repair pathways, enhanced drug reflux within cells, and the inhibition of the cellular response to apoptosis. Previous study has been reported that chemoresistance in cancer therapy is associated with the destabilization or suppression of MOAP-1 and RASSF1A expression[3–5].

MOAP-1 is a member of the paraneoplastic MA antigen (PNMA) family. It was identified due to its association with BAX, a pro-apoptotic protein from the Bcl-2 family, via its BH3-like domain. The BH3-like domain is crucial for facilitating apoptotic signalling by enhancing the interaction with BAX, which triggers the activation and oligomerization of BAX at the mitochondria[6]. RASSF1A is a tumour suppressor gene that has various biological functions, such as regulating BAX-mediated cell death[7].

Previous research has shown that the loss of tumour suppressor proteins like MOAP-1 and RASSF1A in cancer cells is often caused by hypermethylation of their promoter regions. This loss creates conditions that promote tumour growth and resistance to multiple drugs, primarily by halting apoptotic signalling pathways. Thus, the interactions among three tumour suppressors or pro-apoptotic proteins, MOAP-1, Bax and RASSF1A, to form a protein complex that promotes apoptosis signalling could potentially develop sensitized cancer cells by restoring their apoptotic pathways subsequently overcoming their resistance towards conventional chemotherapy.

Novel strategies for genetic material delivery is by employing the use of adenovirus. Adenovirus is among the most promising viral vectors and frequently employed as a viral vector for delivering genetic material to targeted cells, including the delivery of therapeutic gene therapy for treating cancer[8–11]. The introduction of transgenes, specifically those encoding tumour suppressor or pro-apoptotic proteins, into the adenovirus genome facilitated the investigation of protein expression mediated by adenovirus and their corresponding activities in targeted cells[9,12,13].

In this study, AdenoCure 3X consisting a tetracistronic expression construct, Ad-MBRG, encoding adenovirus-mediated expression of tumour suppressor genes of MOAP-1,

Bax, RASSSF1A, and EGFP will be used to restore the apoptotic signaling in cancer cells. This study highlights the optimization in establishing sensitized cancer cells with restored apoptotic signaling mediated by Ad-MBRG in *in vitro* cancer cell model. The development of sensitized cancer cells using adenovirus-mediated expression of tumour suppressor genes in cancer cell lines may serve as a model of experimentation for screening the synergetic efficacy of the anti-cancer compounds.

By integrating gene therapy elements and introduction of anti-cancer compounds in cancer cell lines, this approach presents novel opportunities as an experimental platform for therapeutic interventions. The cancer cell model contributes to the development of novel combination therapies for cancer treatment by identifying potential compounds that target specific vulnerabilities or resistance mechanisms in cancer cells.

2. Materials and Methods

2.1 Materials

The materials employed in this study consist of human cell lines, chemicals, reagents, equipment, apparatus, and consumables. The MOAP-1, Bax and RASSF1A (MBR) recombinant vectors (Ad-MBRG) have been cloned in a polycistronic expression vector and sub-cloned in the adenovirus genome in pShuttle(+) vector as a service provided by Applied Biological Materials Inc (ABM) (Figure 1).

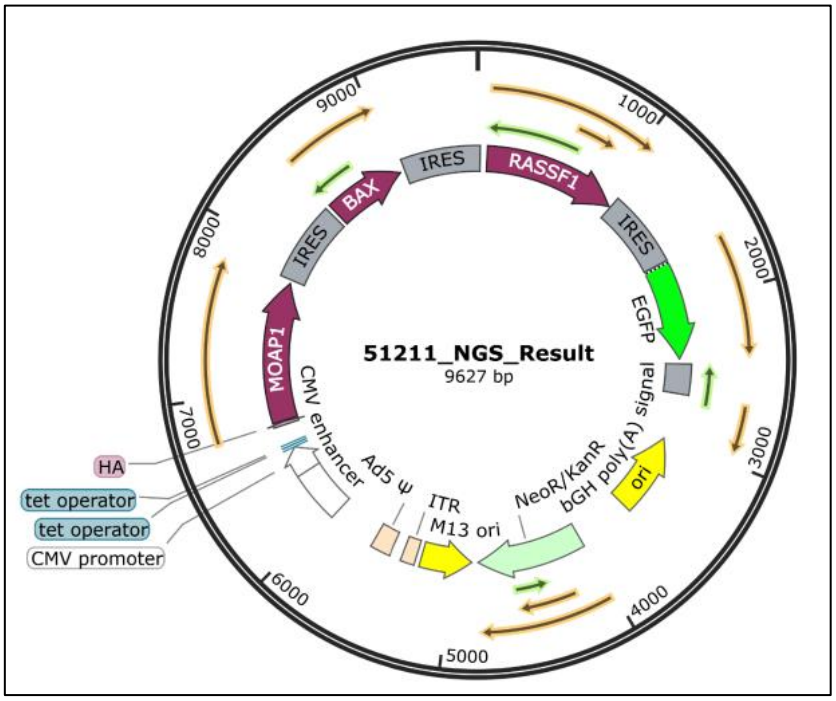

Figure 1. The map of assembly of recombinant pShuttle(+) vector harboring HA-tagged MOAP-1, Bax, RASSF1A and EGFP

2.2 Overview of Methodology

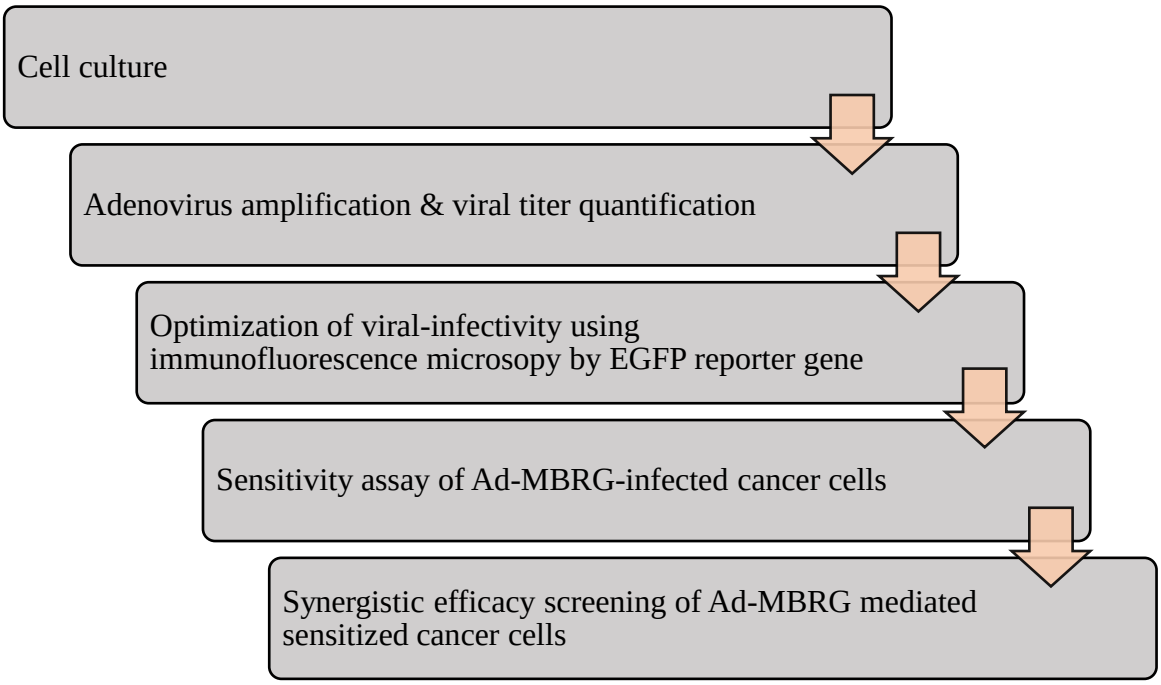

Figure 2. Overview methodology of establishment of sensitized cancer cell model mediated by Ad-MBRG

2.3 Cell Culture

A total of 2 Caucasian cell lines, consisting of MCF-7 Cisplatin-resistant (MCF-7-CR)[14] and HEK 293 (ATCC: CRL-1573) will be cultured in complete DMEM media supplemented (Cat. No.12100–046; Gibco, USA) with 10% FBS (v/v) (Cat. No.10270–106; Gibco, USA), penicillin/streptomycin (100 U/ml) (Cat. No.15140–122; Gibco, USA), MEM non-essential amino acid solution (1X) (Cat. No. 11140–035; Gibco, USA), and L-glutamine (2mM) (Cat. No.25030–081; Gibco, USA). The cell lines will be maintained in a CO_2 incubator at 37°C and 5% CO_2.

2.4 Adenovirus Amplification

Ad-MBRG adenovirus is amplified using human HEK 293 cells to achieve high viral titer (~$1x10^9$ pfu/ml) before being used in subsequent experiments. HEK 293 cells were seeded in 100 mm plates and adenovirus infection assay was performed in complete DMEM media. Cells and media containing adenovirus were harvested after 95% of the cells were detached. The cells were centrifuged at 450 x g for 5 minutes, and the cell pellets were washed with PBS once before resuspended in 1 ml of PBS. Cell lysis was carried out by subjecting the cells to three cycles of freezing at -80°C and thawing at 37°C. The resulting cell debris was then pelleted by centrifugation at 4500 x g for 10 minutes at 4°C. The supernatant which

is now the adenoviral stock were subjected to a final concentration of 10% glycerol (v/v) (Cat. No. 09886-05; Nacalai Tesque, Japan) before long-term storage at -80°C.

2.5 Adenoviral Titer Quantification

The adenoviral stocks were subjected to viral titer quantification using QuickTiter Adenovirus Quantification Kit (Cat. No. VPK 106; Cell Biolabs, USA) according to the manufacturer's instructions. Prior to the quantification of samples, the adenovirus DNA standard curve was generated from the samples provided in the kit. A total of 10 µl of adenovirus stock was diluted to a final volume of 1 ml with 1X PBS supplemented with 10 mM $MgCl_2$ (Cat No. M8266; Merck, USA) and 1 mM $CaCl_2$ (Cat. No. 102378; Merck, USA). Subsequently, the adenovirus stock was subjected to Solution A at 37°C for 30 minutes, and QuickTiter Adenovirus Capture Solution followed by incubation at room temperature on a shaker. The sample was centrifuged, and the pelleted beads were washed twice with Solution B before adding Solution C. Finally, supernatant sample was transferred to 96 well microtiter plate, and subjected to of 1X CyQuant GR Dye followed by fluorescence reading at 480/520 nm filter set. The supernatant samples from the non-infected HEK 293 cells acted as the negative control. The adenovirus titer was calculated based on the standard curve below according to manufacturer's instructions.

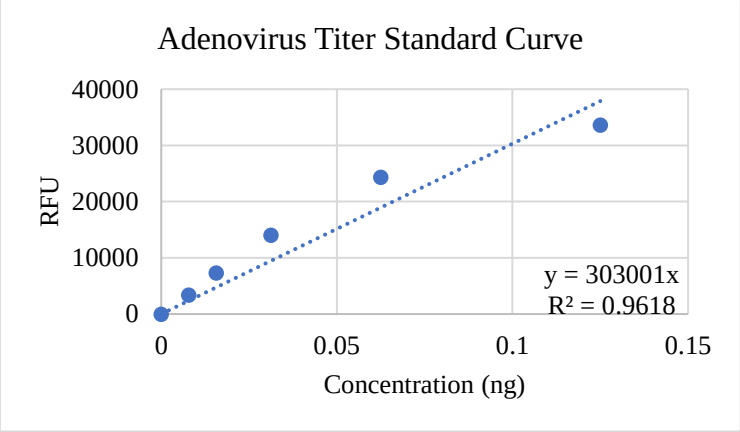

Figure 3. Standard Curve of QuickTiter Adenovirus Titer Quantification

2.6 Optimization of Adenoviral-Infectivity in Cancer Cell Line

The optimal multiplicity of infection (MOI) was determined for MCF7-CR cells. In this respect, protamine sulfate (Cat. No. 29318-54; Nacalai Tesque, Japan) was reported to enhance infection efficiency. The cells were seeded in 6 well plates and a series of MOI concentrations from MOI 0 up to MOI 50 with the addition of 20 µg/ml protamine sulphate was optimized in complete DMEM media for 4 hours. After 4 hours, media containing adenovirus were discarded, and fresh complete DMEM media was added for 24 hours. The

cells were viewed under a fluorescence microscope (Nikon, Japan) according to the manufacturer's protocol, and images were captured using Nikon software. The expression of EGFP signals determined the viral infectivity.

2.7 Visualization of Cell Morphology of Adenoviral-Infected Cancer Cell Line

The cell morphology of adenovirus-infected MCF7-CR cells was visualized under inverted light microscope. The cells were seeded in 6 well plates and a series of MOI concentrations from MOI 0 up to MOI 50 with the addition of 20 μg/ml protamine sulphate was added in complete DMEM media for 4 hours. After 4 hours, media containing adenovirus were discarded, and fresh complete DMEM media was added for 24 hours. The cells were examined with an inverted light microscope following the manufacturer's instructions, and images were acquired using Nikon software.

2.8 Sensitivity of Cancer Cells Towards Ad-MBRG Infection

MCF7-CR cells was seeded in 96-well plates and a series of MOI concentrations from MOI 0 up to MOI 50 with the addition of 20 μg/ml protamine sulphate was optimized in complete DMEM media for 4 hours. After 4 hours, media containing adenovirus were discarded, and fresh complete DMEM media was added for 24 hours. The cells were subjected to MTT reagent (Cat. No. 475989; Merck, USA) at 5mg/ml concentration and the formazan crystal was dissolved in 100 μl of DMSO (Cat. No. 1029522511; Merck, USA) per well. The absorbances were measured at 570 nm with a microplate reader (Tecan, Switzerland) and the reading was normalized against non-infected control.

2.9 Drug Sensitivity of Ad-MBRG-Infected Cancer Cells Towards Natural Derived Compounds

MCF7-CR cells were seeded in 96-well plates at 70% confluency. MOI 5 is optimum for this study where most of the cells expressed EGFP signals which indicated successful adenovirus infection and the low MOI is used to avoid the trigger of immune response. To infect the cells, the cells were subjected to infection at MOI 5 for 4 hours with 20 μg/ml protamine sulphate at 37°C. After 4 hours, the media containing the adenovirus were discarded. The infected cells was subsequently treated with natural derived compounds, piperonal (Cat. No. 120-57-0; Merck, USA), pyrazole (Cat No. 288131; Wako Pure Chemicals, USA) and morin (Cat. No. 654055013, Fluka Chemicals, Switzerland) at a serial of dilution up to 4000 μg/ml, respectively. The cells were harvested at 24, 48, and 72 hours, respectively and subjected to MTT assay at 5 mg/ml concentration. The formazan crystal was dissolved in 100 μl of DMSO and the absorbances were measured at 570 nm with a microplate reader. The reading was normalized against non-infected and non-treated control.

2.10 Statistical Analysis

The data were presented as the mean ± standard deviation (SD) of three independent experiments (n=3) or as indicated. GraphPad Prism 10 were utilized for data analysis. The Student's *t*-test was utilised to assess the statistical significance of the observed data variations, indicated by the *p*-value of $p<0.001$.

3. Results and Discussions

Conventional chemotherapy remains a mainstay in cancer treatment; however, the emergence of chemo-resistant tumours necessitates the development of novel therapeutic strategies. To address the growing challenge of chemoresistance, this study presents a novel approach for developing a sensitized cancer cell model using AdenoCure 3X to accelerate the discovery of novel drugs. We constructed a tetracistronic adenoviral vector (Ad-MBRG) expressing tumour suppressor genes MOAP-1, Bax, and RASSF1A, which are frequently epigenetically silenced in cancer and contribute to apoptotic dysfunction. This study explored the potential of adenovirus-mediated restoration of apoptotic signaling pathways to establish a sensitized cancer cell model suitable for anti-cancer drug screening.

To begin with the characterization of the recombinant adenovirus, the adenovirus particles was amplified in HEK 293 cells and the viral titer was quantified using commercially available assay kit. This step ensures a sufficient and well-characterized supply of adenoviral particles for subsequent biological experiments. The adenovirus-mediated expression of Ad-MBRG was subsequently characterized through several biological assays to determine its viral infectivity in developing a sensitized cancer cell line.

For instance, the adenovirus was characterized by its ability to infect cancer cell line by optimizing the MOI, defined as the ratio of infectious virions to cells. The MOI was determined for optimum infectivity in cancer cell line by performing fluorescence microscopy by detecting the EGFP expression as the adenovirus construct was tagged with EGFP to indicate successful infections in cancer cell line.

As shown in Figure 4, the EGFP was expressed minimally at MOI 1, and the expression increased corresponding to the increase of MOI. Interestingly, the EGFP expression was qualitatively reduced at MOI 50 in infected cells due to cell death mediated by Ad-MBRG. This suggests that the EGFP expression will be diminished as the cells are non-viable.

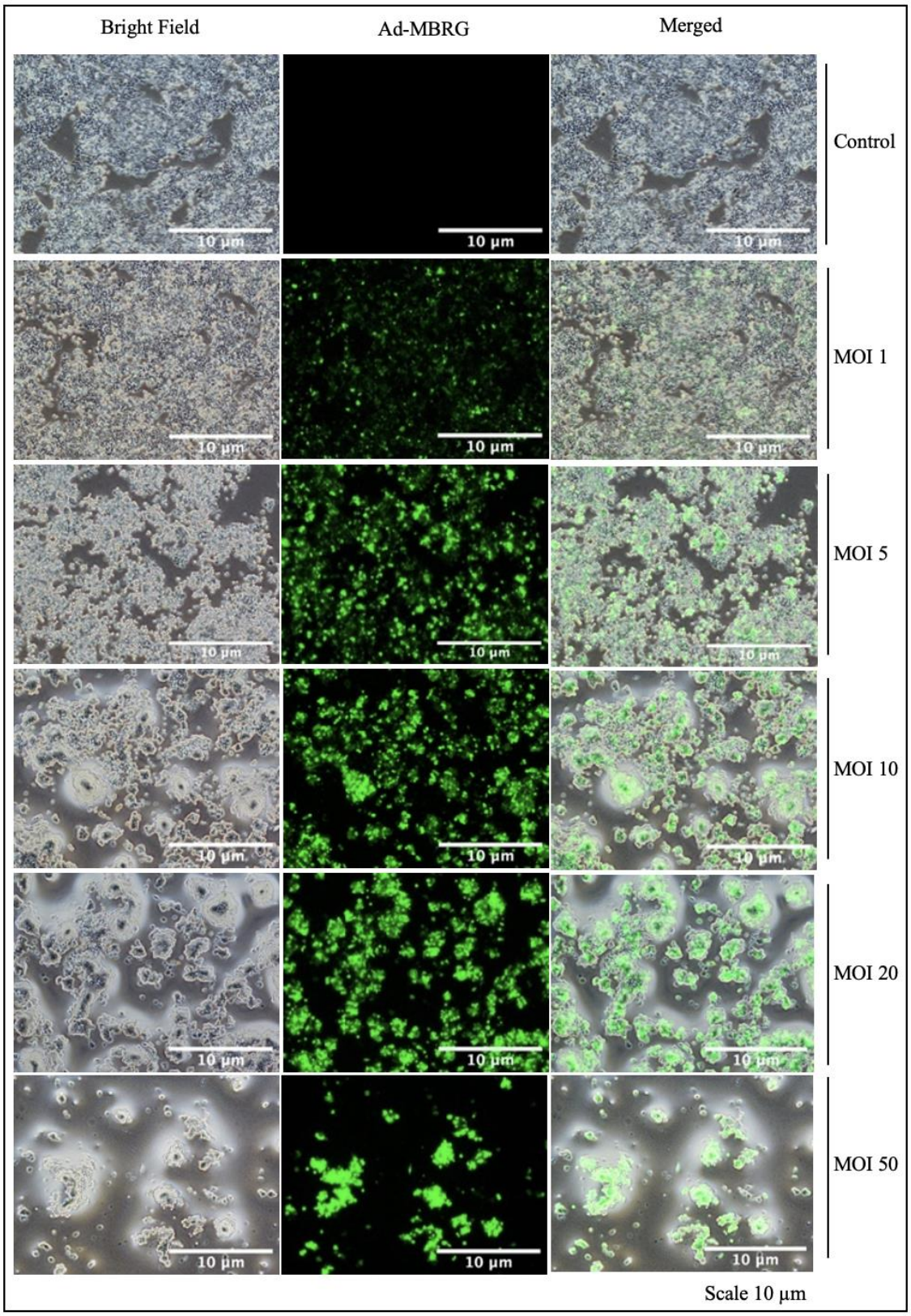

Figure 4. Adenovirus infectivity of MCF7-CR cells at a series of MOI 0 to 50 for 24 hours.

Moreover, it has been established that external stimuli influence the structure, size, density, and surface of cellular components, among other morphological attributes of cells[15].

With respect to this, the cells were subjected to adenovirus infection at a range of MOI 1 to 50 and subsequently examined under microscopy to determine the morphology changes.

As shown in Figure 5, the adenovirus-infected cells demonstrated morphology changes even when low MOI was applied. For instance, MOI 5 of the infected MCF7-CR cells showed rounded morphology and started clumping compared to the control cells, where most of the transparent cells are attaching well with normal and elongated morphology. Furthermore, the clumping of the rounded cells is significantly observed at adenovirus-infected cells at MOI 10 onwards due to cell death mediated by Ad-MBRG.

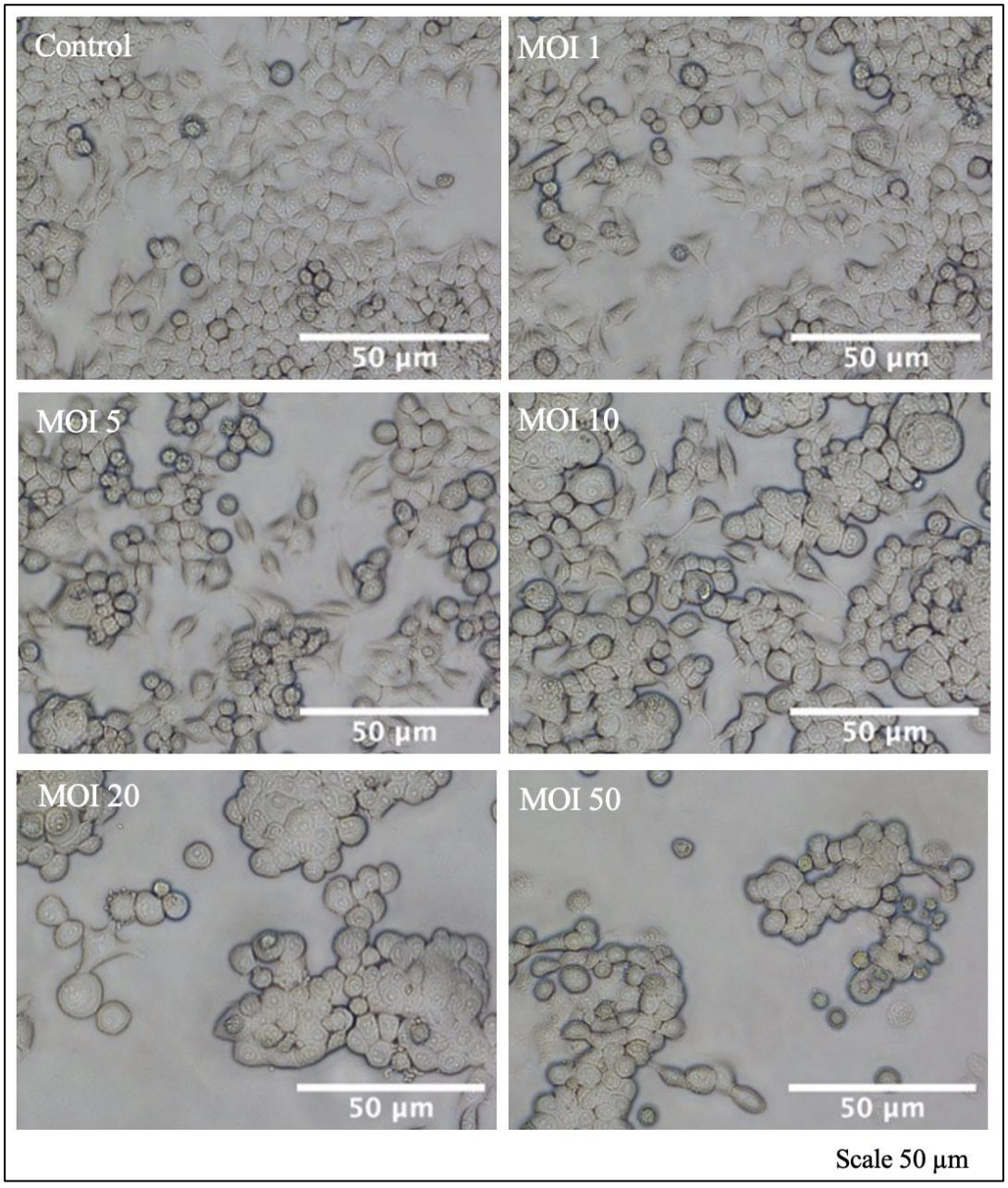

Figure 5. Bright field images of Ad-MBRG-infected MCF7-CR cells at a series of MOI 0 to 50 for 24 hours.

Next, the sensitivity of Ad-MBRG-infected MCF7-CR cells compared to non-infected controls is assessed. This includes determining the optimal MOI while minimizing cytotoxicity. The sensitivity of Ad-MBRG in cancer cells is investigated by applying different MOI of Ad-MBRG followed by measurement of cell viability. As shown in Figure 6, Ad-MBRG showed MOI-dependent reduction of cell viability in the cancer cells where the cell viability decreased gradually, corresponding to the increase of MOI. Interestingly, the Ad-MBRG-infected MCF7-CR cells demonstrated a significantly reduced cell viability compared to the non-infected control at $p<0.001$.

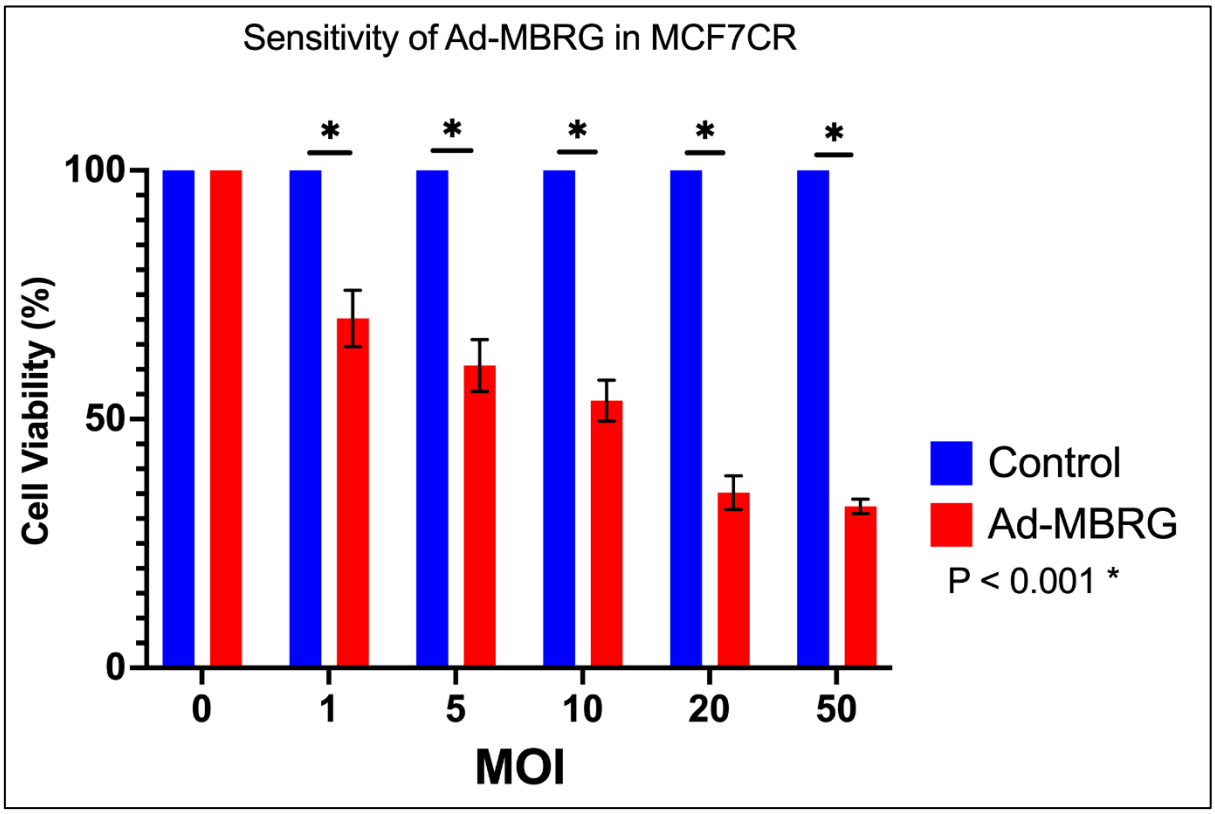

Figure 6. Sensitivity of cancer cell towards adenoviral infection mediated by Ad-MBRG. The cancer cells were infected with a series of MOI of Ad-MBRG followed by measurement of cell viability at 24 hours.

The sensitivity of Ad-MBRG to cancer cells demonstrated in Figure 6 suggested that adenovirus infection induced oncolysis or cell death of cancer cells which is was similarly reported by other investigators[16]. In addition, the cell death mediated by Ad-MBRG was likely due to the overexpression of tumour suppressor genes to form a protein complex that promotes apoptosis signalling[17,18].

As reported in a previous study, MOAP-1 is associated with BAX through its BH3-like domain. The BH3-like domain is crucial for facilitating apoptotic signalling by enhancing the interaction with BAX, which triggers the activation and formation of BAX oligomers at the mitochondria[6]. The BH3 domain promotes the activation of BAX by

changing its conformation leading to its exposure and resulting in the engagement of the BAX monomer[19]. BAX confirmation changes allow the C-terminus of BAX to bind to the mitochondria membrane, anchoring BAX oligomers to mitochondria, resulting in mitochondria dysfunction and the release of mitochondrial cytochrome C, ultimately leading to apoptosis[20]. Hence, the decrease in cell viability observed in Ad-MBRG-infected cells in Figure 6 is probably caused by the reactivation of apoptotic signalling mediated by Ad-MBRG.

To study the sensitization of cancer cells mediated by Ad-MBRG infections, the infected cancer cells were subjected to treatments using natural derived compounds, such as piperonal, pyrazole and morin. While natural product-derived compounds have shown promise in pre-clinical studies, their efficacy as single agents for cancer treatment is often limited[21]. This necessitates their exploration in combination with conventional chemotherapy regimens or immunotherapy. These combinations can exploit synergistic effects, enhancing the overall efficacy of treatment while potentially reducing the dosage of chemotherapeutic drugs, thereby mitigating associated toxicity and side effects[21,22].

In addition to assessing the sensitivity of cancer cell towards adenoviral infection, the strategy of treatment consist of synergistic efficacy screening on this sensitized cancer cells with treatment using natural derived compounds. As shown in Figure 7, the sensitization of MCF7-CR cells mediated by Ad-MBRG infection were demonstrated against piperonal, pyrazole and morin resulting in significantly lower IC 50 values as compared to treatment of natural derived compounds alone.

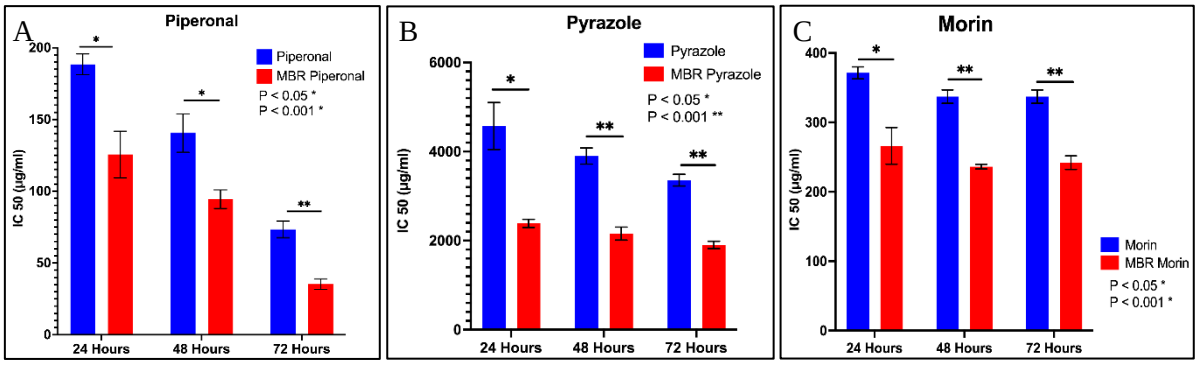

Figure 7. Sensitization of MCF7-CR cells mediated by Ad-MBRG infection against A) piperonal, B) pyrazole and C) morin. The cancer cells were infected with Ad-MBRG at MOI 5, subsequently subjected to respective treatments, followed by measurement of cell viability at 24, 48 and 72 hours.

The drug sensitivity shown in Figure 7 demonstrate the synergetic effect of the adenovirus based cancer gene therapy using Ad-MBRG with the presence of apoptotic stimuli from the natural derived compounds. These apoptosis stimuli were reported to

promote activation of MOAP-1, Bax, and RASSF1A, which were reported to exist as a 'Zymogen' or inactivated state that requires activation by apoptotic stimulus, mainly through receptor-mediated activation of cascading cellular signalling events.

For instance, MOAP-1, a BH3-like protein, is a downstream mediator of RASSF1A, which promotes BAX activation and cell death during apoptosis[23]. Recent data demonstrate that MOAP-1 and RASSF1A are associated with TNFR in response to TNF stimulation, initiating a signalling cascade in which activated MOAP-1 promotes BAX translocation to mitochondria, leading to the release of cytochrome C from mitochondria[7,19,24–26]. Therefore, MOAP-1 and RASSF1A serve a crucial function in mitochondrial-dependent apoptosis by regulating and preserving BAX activation leading the cells to be responsive to death signals mediated by external and internal apoptotic pathways[27,28].

Taken together, this Ad-MBRG-mediated sensitized cancer cell model presents a promising *in vitro* tool for screening both natural and synthetic anti-cancer compounds. The re-activation of the apoptotic signaling pathway through the upregulation of tumour suppressor genes like MOAP-1, Bax, and RASSF1A in these sensitized cells enhance the screening of anti-cancer compounds. Ultimately, this experimental model has the potential to significantly accelerate the discovery of novel drugs for chemo-resistant cancers.

4. Conclusion

Our study demonstrates that Ad-MBRG infection can potentially reactivate the dysfunctional apoptotic signaling pathway in cancer cells, thereby overcoming their resistance to conventional chemotherapy. This work outlines a novel approach for establishing a sensitized cancer cell model mediated by Ad-MBRG, which holds promise for accelerating the discovery of novel therapeutic agents for chemo-resistant cancers. Here, we detail the steps involved in generating this sensitized cell line, including:

1) **Cell Line Initiation:** To initiate the cell culture of a selected cell line for the drug study.

2) **Adenovirus Amplification and Quantification:** This section outlines the methods for amplifying and quantifying adenoviral particles for use as a stock reagent.

3) **Optimization of Adenoviral Infectivity:** We detail the optimization process for achieving efficient viral infection and determine the optimal multiplicity of infection (MOI) for the chosen cell line.

4) **Characterization of Ad-MBRG in the Selected Cell Line:** This section describes the characterization methods employed to evaluate the efficacy of Ad-MBRG by assessing the sensitivity of the infected cell line.

5) **Drug Sensitivity of the Established Sensitized Cancer Cell Model:** We outline the methods used to assess drug sensitivity in the established sensitized cell line, aiming to identify novel drugs with potential synergistic effects with adenovirus-based cancer gene therapy.

In conclusion, establishing a sensitized cancer cell model utilizing AdenoCure 3X, mediated by Ad-MBRG could potentially serves as a valuable model of experimentation for screening the synergetic efficacy of the anti-cancer compounds. By integrating gene therapy elements and introducing anti-cancer compounds into cancer cells, this novel approach offers promising opportunities for therapeutic interventions. The utility of this model is further supported by pending Malaysia Utility Innovation Application (No. UI2024003814). Future studies will explore the broader applications of this sensitized cancer cell model in drug discovery.

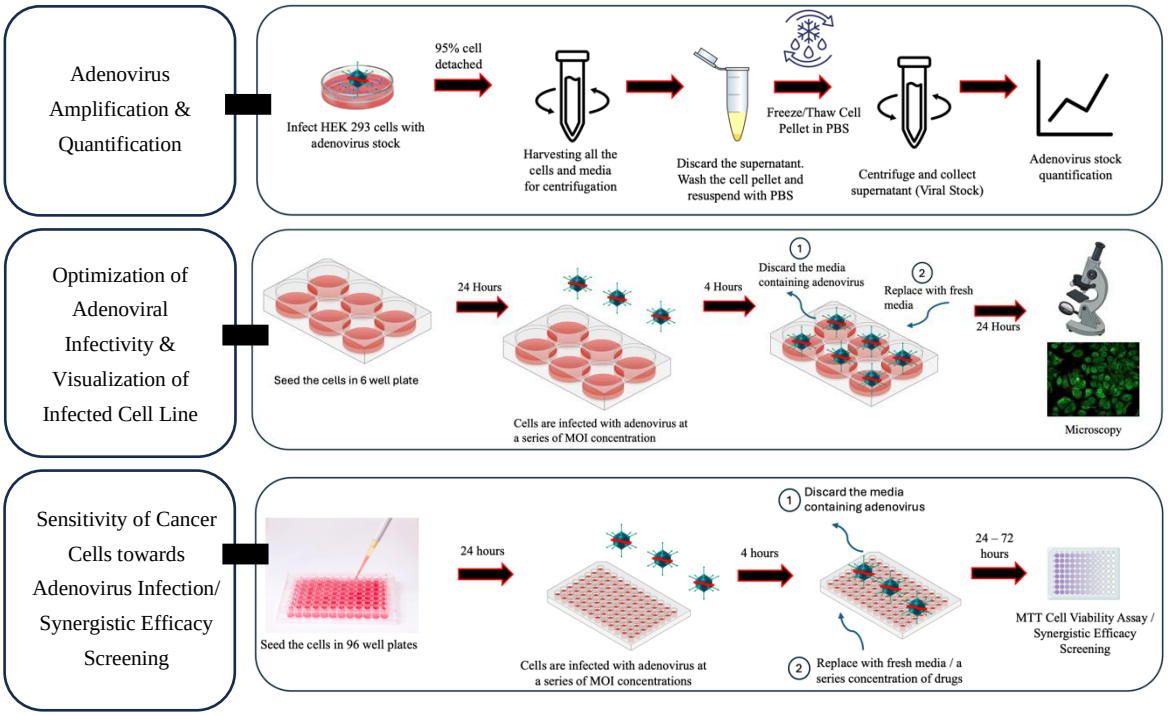

Figure 8. Graphical summary of establishment of adenovirus-infected cancer cell as a sensitized cancer cell model for drug discovery

Author Contribution: The authors confirm their contribution to the paper as follows: study conception and design: B.H.G., K.O.T., and E.W.T. Data collection: E.W.T. Draft manuscript preparation: E.W.T, and B.H.G. All authors reviewed the results and approved the final version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest to this work.

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