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Establishment of Adaptive Drug-resistant Colorectal Cancer Cell Model

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Abstract: Colorectal cancer (CRC) ranks among the most prevalent cancers and contributes significantly to cancer-related fatalities. Chemoresistance in CRC poses a considerable therapeutic challenge, underscoring the need to comprehend the underlying mechanisms for the development of alternative strategies to overcome this resistance. Utilizing isogenic cell lines with acquired drug resistance is one of the prominent experiment approaches for studying chemoresistance, enabling the exploration of adaptive cellular responses to chemotherapeutic agents that confer resistance in cancers. However, establishing such cell models is challenging, and there are limited readily available protocols for scientists as references. This paper aims to elucidate the methodology for establishing a laboratory isogenic adaptive chemoresistant cell model, focusing on a cisplatin-resistant CRC cell model. From a panel of three human CRC cell lines, HCT116 was selected as the parent cell line due to its high cisplatin sensitivity. HCT116 cells were subjected to pulsed or continuous cisplatin treatments that resulted in successful selection of seventeen HCT116 sublines that exhibited varying degrees of cisplatin resistance. Only one HCT116 subline with transient acquired cisplatin resistance was established using pulsed exposure method while the method involved continuous cisplatin treatment has successfully established two resistant lines HCT116/I24781 and HCT116/I248 demonstrate 24.5-fold and 19.2-fold resistance respectively. These resistant cells showed significantly reduced growth rate with slight change in cell morphology and relatively stable resistance that remained unaffected for 8 continuous passages in cisplatin-free environment and 2 cryopreservation cycles. Together, these results suggest that continuous exposure with stepwise dose increase of drug is promising for establishing adaptive drug-resistant cell lines with significant drug resistance.

Keywords: Chemoresistance; colorectal cancer; cisplatin; cell culture; drug resistance

1. Introduction

As the third most prevalent cancer globally, colorectal cancer (CRC) accounted for nearly 10% of all diagnosed cancers worldwide in 2020[1]. Despite significant advancements in cancer research over the past decades, the mortality rate of CRC remains high, with CRC ranking as the second leading cause of global cancer mortality, responsible for 9.4% of all cancer-related deaths[1]. Chemotherapy resistance, or chemoresistance, is a major hurdle in the management of CRC, contributing to the stagnant 5-year survival rate of advanced CRC, ranging from 10% to 20%[2,3]. Therefore, understanding the molecular mechanisms underlying chemoresistance in CRC is crucial for developing effective strategies to overcome the limitations of current chemotherapy regimens.

Chemoresistance in CRC is a multifactorial phenomenon driven by variations in drug targets or cancer types, leading to the emergence of diverse drug resistance pathways. For instance, cancers resistant to the two commonly used chemotherapeutic agents, cisplatin and cyclophosphamide, exhibit distinct resistance mechanisms. Cisplatin resistance is often linked to increased activity of the copper ion transporter[4], while cyclophosphamide resistance was hypothesised to involve glutathione-mediated detoxification and DNA damage repair[5]. Despite current CRC treatment protocols employing combination therapies consisting of at least two different chemotherapy agents, chemoresistance remains a persistent challenge complicating the management of CRC[2,3,6,7]. Importantly, CRC patients that were unresponsive to FOLFOX or CAPOX combination therapy regimens often display strong oxaliplatin resistance[8]. This underscores the importance of platinum resistance in multidrug resistance (MDR) phenotype observed in CRC.

Laboratory-based chemoresistant cell models serve as invaluable biological model for researchers, enabling the exploration of chemoresistance mechanisms and the development of alternative strategies to overcome the chemoresistant tumours. Establishing appropriate laboratory cell models for studying chemoresistance in CRC is essential to ensure the clinical relevance of experimental outcomes. Chemoresistance in cancers can be broadly categorized into two main types: *de novo* resistance and adaptive or acquired drug resistance. Due to the heterogeneity of cancer tumours, *de novo* drug-resistant cancer cells are inherently impervious to chemotherapy that are present with the tumour from the outset[9]. These *de novo* chemoresistant cells contribute to minimal residual disease, leading to recurrent malignancies that exhibit high resistance to chemotherapy[10]. On the other hand, cancer cells with acquired drug resistance were initially susceptible to the cytotoxic effects of the treatment but subsequently developed counteracting mechanisms after prolonged exposure to chemotherapy[11]. Understanding chemoresistance in cancer cells with adaptive drug

resistance is of paramount importance, as it sheds light on the differentially regulated biological processes that confer a chemoresistant phenotype.

Isogenic adaptive drug-resistant cell lines are typically established by applying selective pressure to a parent cell line that exhibits strong sensitivity to the drug of interest. The term "parent cell line" refers to a foundational group of cells that serves as the origin for the development of novel cellular models. Through exposure to escalating concentrations of the drug, the parent cell line undergoes selective pressure, allowing only those that have acquired resistance traits to survive. These surviving cells then become the cornerstone for generating isogenic drug-resistant cell lines. This strategy ensures genetic homogeneity, enabling a focused exploration of the specific mechanisms underlying drug resistance within a controlled and reproducible experimental framework.

To date, a standardized protocol for the establishment of an isogenic adaptive drug-resistant cancer cell line remains elusive. Given the implication of platinum resistance in CRC chemoresistance[8], this paper describes the successful establishment of isogenic adaptive cisplatin-resistant CRC cell lines. Beyond CRC and cisplatin resistance, the methods presented in this paper can serve as a valuable reference for researchers aiming to establish isogenic drug-resistant cancer cell models.

2. Materials and Methods

2.1 Materials

Human CRC cell lines, HCT116, SW480 and HT29 were obtained from the American Type Culture Collection (ATCC, USA). Roswell Park Memorial Institute cell culture medium (RPMI-1640) was procured from Sigma (USA) and were supplemented with 10% foetal bovine serum (FBS) purchased from Thermo Fisher (USA) and 1% penicillin/streptomycin from Nacalai Tesque (JP). 25 g/L trypsin/ethylenediaminetetraacetic acid (trypsin/EDTA) was obtained from Nacalai Tesque (JP). Cisplatin and dimethyl sulfoxide (DMSO), Hybri-Max DMSO, trypan blue and Nalgene® Mr. Frosty were procured from Sigma while 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Nacalai Tesque (JP). Cell culture consumables (cell culture flasks, plates, cryovials) were purchased from SPL (KR).

2.2 Methods

The general workflow for the establishment of isogenic adaptive drug-resistant cancer cell lines was illustrated in Figure 1. The process typically involves three steps:

1. Selection of parent cell line.

2. Conditioning/Selection of adaptive drug-resistant cells.
3. Validation of adaptive drug-resistant cell lines.

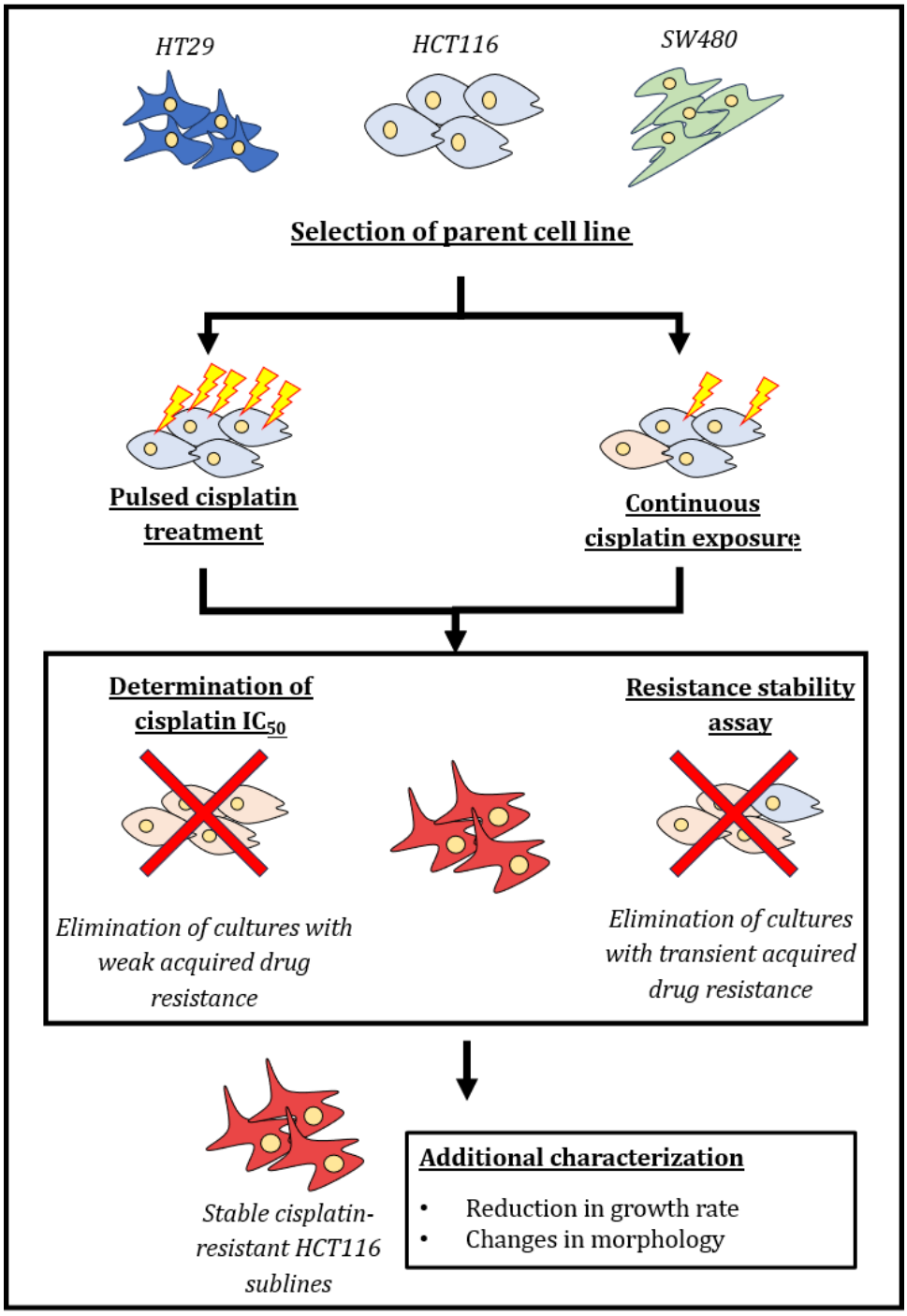


Figure 1. General workflow diagram for the establishment of laboratory isogenic adaptive drug-resistant cell lines for chemoresistance studies.

2.3 Cell Culture

All cells were cultured in complete RPMI-1640 medium as adherent cells in a 37°C incubator supplemented with 5% CO_2. The cells were passaged at 70% to 80% confluency.

The sub-confluent cells were detached using 25 g/L trypsin/EDTA for 10 minutes before neutralizing the trypsin with equal volume of complete medium. The cell suspension was collected and the detached cells were pelleted through centrifugation at 1000 RPM for 8 minutes. Cell pellet was resuspended in fresh complete RPMI-1640 medium and 5% to 10% of the cells were transferred to a new flask to continue the culture.

2.4 Selection of Parent Cell Line

The sensitivity to cisplatin treatment of the human CRC cell lines was studied by elucidating the 50% inhibitory concentration (IC_{50}) of cisplatin using MTT cell viability assay. The cells were seeded onto 96-well plates and allowed adhering for 24 hours. Old culture medium was replaced with fresh complete RPMI-1640 medium supplemented with various working concentrations of cisplatin. The plates were returned to incubator for 24 or 48 hours. At the end point of treatments, cisplatin-supplemented medium was replaced with fresh complete medium with 0.5 mg/mL MTT and the plates were returned to 37°C incubator for 3 hours. Following incubation, MTT-supplemented medium was aspirated and the formazan crystals were dissolved in DMSO. Absorbance was measured at 570 nm using Tecan Plate Reader Infinite Pro 200. The assay was performed in triplicates and three independent experiments were performed. Cell viability was calculated using following equation:

$$Cell\ viability\ (\%) = \frac{Absorbance\ of\ treated\ cells}{Absorbance\ of\ control\ cells} \times 100\%$$

IC_{50} concentrations of cisplatin was calculated by interpolating the survival curve at 50% cell viability. Cell line with the strongest sensitivity to cisplatin treatment was selected as parent cell line for the establishment of isogenic adaptive cisplatin-resistant cell models.

2.5 Establishment of Adaptive Cisplatin-Resistant HCT116 Sublines

Cisplatin-sensitive CRC cell line, HCT116 was selected for the establishment of adaptive cisplatin-resistant CRC cell lines. HCT116 cells were subjected to “Pulsed” or “Continuous” cisplatin exposure. Constant or stepwise increase in cisplatin concentration strategies were also employed. Together, there are four different drug exposure strategy in total for the establishment of adaptive drug resistant cell lines, “pulsed exposure with constant dosing”, “pulsed exposure with stepwise dosing increase”, “continuous exposure with constant dosing” and “continuous exposure with stepwise dosing increase”. As risk management, comparative selection strategy was used throughout the selection process.

For “Pulsed” exposure, HCT116 cells were seeded into T25 flasks and grew to 70% confluency before treated with at least 15 μM cisplatin for 24 hours. Treatments were then

removed and the surviving cells were cultured in cisplatin-free complete RPMI-1640 medium until 90% confluency. The sub-confluent cultures were then divided equally into two new T25 flasks and grew to 70% confluency. One of the cultures was subjected to cisplatin treatment with unchanged concentration while the other culture was treated with increased cisplatin concentration. Throughout the experiment, duration of cisplatin treatment remained unchanged as 24 hours for all lineage of cells. These steps were repeated and cultures that survived 10 pulsed cisplatin treatments were selected.

For "Continuous" exposure, HCT116 cells were maintained in complete RPMI-1640 medium supplemented with either 2 μM or 3 μM cisplatin until 80% confluency. Each sub-confluent cultures were then passaged and divided equally into two new T25 flasks where one of the cultures was maintained in complete medium with same cisplatin concentration while the other flask of cells was cultured in complete medium with increased cisplatin concentration. This step was repeated and cells that survived 10 continuous passages in cisplatin-supplemented complete medium were selected.

Selected adaptive cisplatin-resistant HCT116 sublines were maintained in cisplatin-free medium following the protocol described in previous section for at least 2 passages before any experiments. Changes in cisplatin IC_{50} compared to parent HCT116 cells were determined using MTT assay. The resistance phenotype was expressed by fold resistance and was calculated using following equation:

$$Fold\ resistance = \frac{Cisplatin\ IC_{50}\ of\ resistant\ subline}{Cisplatin\ IC_{50}\ of\ HCT116}$$

2.6 Resistance Stability Assay

Selected adaptive cisplatin-resistant HCT116 sublines were subjected to two assays to assess the stability of their resistance phenotype

2.6.1 Freeze-thaw assay

Resistant cells were cryopreserved in cryopreservation medium of 90% FBS and 10% Hybri-Max DMSO. Cells were stored in cryovials placed in Nalgene® Mr. Frosty and stored in -80°C for 24 hours before transferred to liquid nitrogen storage for another 48 hours. After that, the cells were thawed in 37°C water bath for 90 seconds. Equal volume of pre-warmed complete RPMI-1640 medium was added to the cell suspension slowly. The cell suspension was then transferred to a 15 mL centrifuge tube and centrifuged at 1000 RPM for 5 minutes. The supernatant was discarded and the cells were resuspended in fresh complete RPMI-1640 medium before transferred to a cell culture flask. The cells were maintained in cisplatin-free complete RPMI-1640 medium for 2 passages before assessed for cisplatin IC_{50} using MTT

assay. This freeze-thaw cycle was repeated again and cisplatin IC_{50} was also determined after second freeze-thaw cycle.

2.6.2 Cisplatin withdrawal assay

Resistant cells were maintained in cisplatin-free complete RPMI-1640 medium for 8 continuous passages following protocols described in previous sections without any additional exposure to cisplatin. Changes in cisplatin IC_{50} (if any) were investigated using MTT assay.

2.7 Growth Rate Assay

HCT116 and its adaptive cisplatin-resistant sublines were seeded onto 6-well plates at 1.25 x 10^5 cells/well and incubated in 5% CO_2 incubator at 37°C for 24, 48 or 72 hours. Following incubation, the cells were detached and trypan blue exclusion assay was performed to determine the total number of cells at each respective time points. Cells were mixed with 0.4% trypan blue solution and the cells were counted using a haemacytometer under inverted light microscope. Growth rate and doubling time of the cells were determined using the following equations:

$$Growth\ rate = \frac{ln\ ln\ (\frac{Cell\ number\ at\ each\ time\ points}{1.25\ \times\ 10^5})}{Incubation\ time\ (h)}$$

$$Doubling\ time\ (h) = \frac{ln\ ln\ 2}{Growth\ rate}$$

2.8 Visualization of Cell Morphology Using Inverted Light Microscopy

HCT116 and its adaptive cisplatin-resistant sublines were seeded onto 6-well plate and allowed attachment for 48 hours. Cellular morphology was examined using DS-5Mc-U2 microscope (Nikon, JP). Images at 200x magnifications were captured and viewed using NIS Elements Viewer Software (v5.21, Nikon, JP).

2.9 Statistical Analysis

Student's *t*-tests were performed when comparing between 2 groups while one-way ANOVA with multiple comparisons and Tukey's *post hoc* (p.adj < 0.05) was used when comparing one variable among 3 or more groups. All experimental data were presented as mean ± standard deviation obtained from three independent experiments. All statistical analyses were performed using GraphPad Prism Software v9.3.1.

3. Results and Discussions

It is inherently important to select a stable cell line that responds well to the treatment of candidate drug-of-interest for the establishment of adaptive drug-resistant isogenic sublines[12]. From a panel of three CRC cell lines, cisplatin sensitivity was compared by analyzing cisplatin IC_{50} values. HCT116 consistently exhibited the strongest cisplatin sensitivity following both 24-hour and 48-hour treatments (Figure 2). Therefore, HCT116 was chosen as the parent cell line for establishing adaptive cisplatin-resistant CRC cell lines.

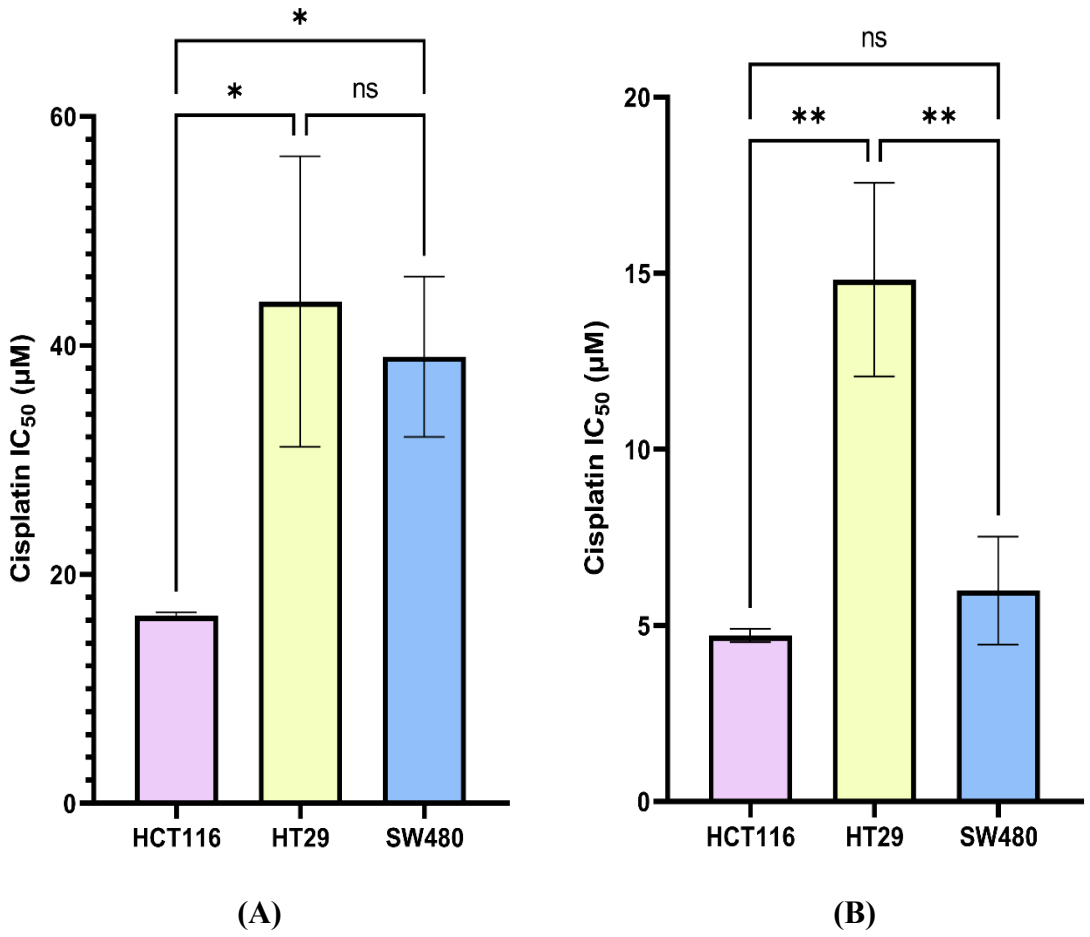


Figure 2. Comparison of cisplatin IC_{50} concentrations in HT29, HCT116 and SW480 cells. (A) 24-hour cisplatin treatment. (B) 48-hour cisplatin treatment. Data represents mean ± standard deviation from three independent experiments (n=3). ** p<0.01; *** p<0.001; **** p<0.0001.

Establishment of isogenic cell line with acquired drug resistance is of paramount importance for studying the underlying mechanisms that confer chemoresistance in cancers. This model offers more relevant and less variable observation by eliminating genetic variations, in contrast to studies employing non-isogenic cell lines for comparison. There are two main types of adaptive drug-resistant cell lines, the clinically relevant and the high-level

laboratory model. Different drug exposure methods are needed for the development of each of these cell models: pulsed or continuous exposure.

Clinically relevant adaptive drug-resistant cell lines are developed to mimic patients undergoing chemotherapy. Therefore, pulsed exposure to the drug was often employed and the surviving cells were allowed to propagate in drug-free medium before next treatment cycle. Although the resulting cell lines are more clinically relevant, there are several disadvantages to using this cell model for research. Clinically relevant drug-resistant cell models usually exhibit relatively low resistance, typically in the range of 2- to 5-fold, and they tend to be unstable[12]. Additionally, the detectable molecular changes in this cell model are usually relatively small.

In contrast, high-level laboratory models typically demonstrate more stable resistance and exhibit more pronounced molecular changes, making them better suited for studying resistance-related molecular mechanisms. High-level drug-resistant cell models are established through continuous drug exposure. Continuous drug exposure exerts high selective pressure on the parent cell line, allow for stricter selection of cell populations that adopted counteracting mechanisms against drug of interest. However, despite the advantages, establishing high-level laboratory models can be considerably more challenging as the cells are continuously exposed to compounds which they are highly sensitive to. It's important to note that while high-level laboratory models offer certain advantages, they may not be as clinically relevant as the previous model, given the continuous exposure requirement.

Besides exposure, dosage is also a crucial factor to consider when establishing an adaptive drug-resistant cell line. Initially, HCT116 cells underwent two treatment cycles of various combinations of exposure methods and dosages to determine the suitable starting point that HCT116 cells would survive (Figure 3). 11 different drug exposure strategies were tested, and HCT116 cells only survived two pulsed treatments and two continuous treatment strategies (Figure 3). Consequently, these treatment strategies were employed to establish adaptive cisplatin-resistant HCT116 sublines, utilizing a comparative selection strategy with stepwise dose increase.

Although increasing selective pressure could result in the selection of cell lines with stronger acquired drug resistance, this approach could also lead to a significantly higher risk due to two different reasons:

1. Cell lines that are still undergoing drug exposure cannot be cryopreserved, as they may not survive cryopreservation or the acquired drug resistance could diminish due to cryopreservation stress.

2. Cells behave unpredictably when under prolonged cytotoxic stress, and slight changes in dosing may have a detrimental effect on cell viability.

Therefore, comparative selection strategy is an important risk management approach, especially when a stepwise increase of drug dosage is employed. At the end of each treatment cycle, the sub-confluent cells that survived the current treatment cycle were equally divided into two different cultures, of which only one culture was exposed to an increased drug dosage. This approach enables us to apply stronger selective pressure with decreased risk, as only half of the culture was exposed to an increased drug dosage.

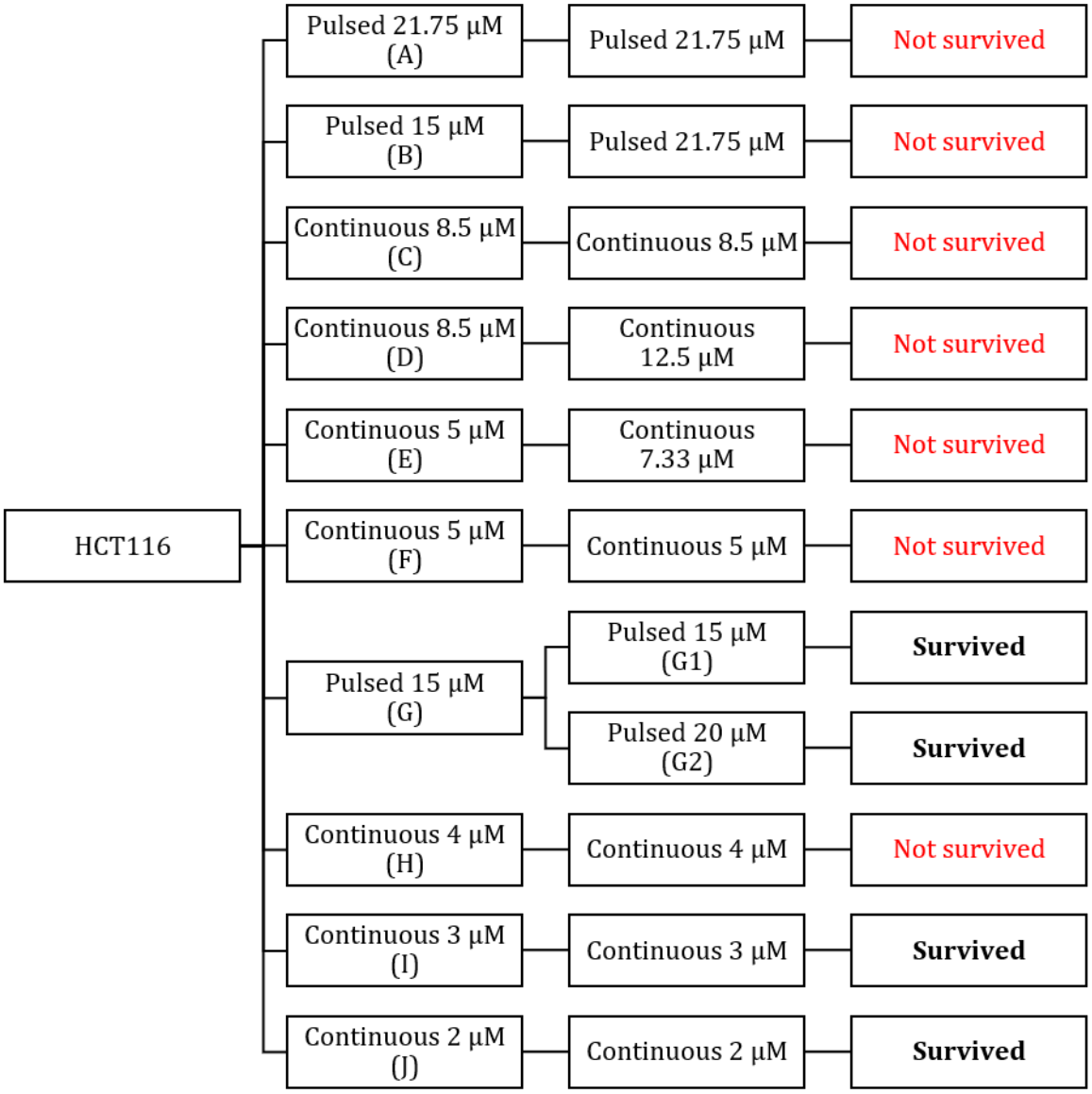
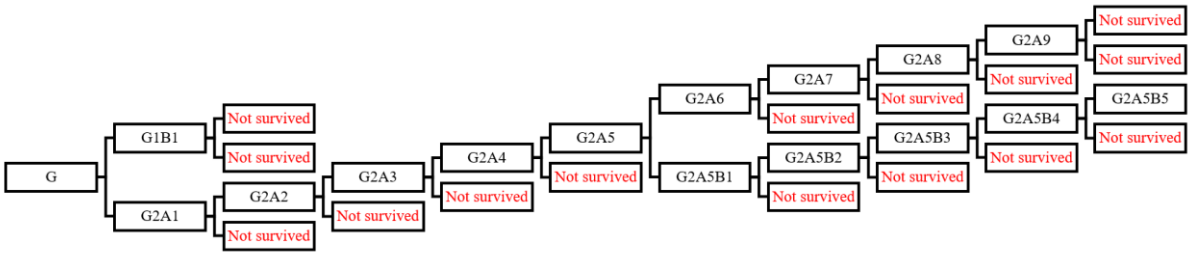
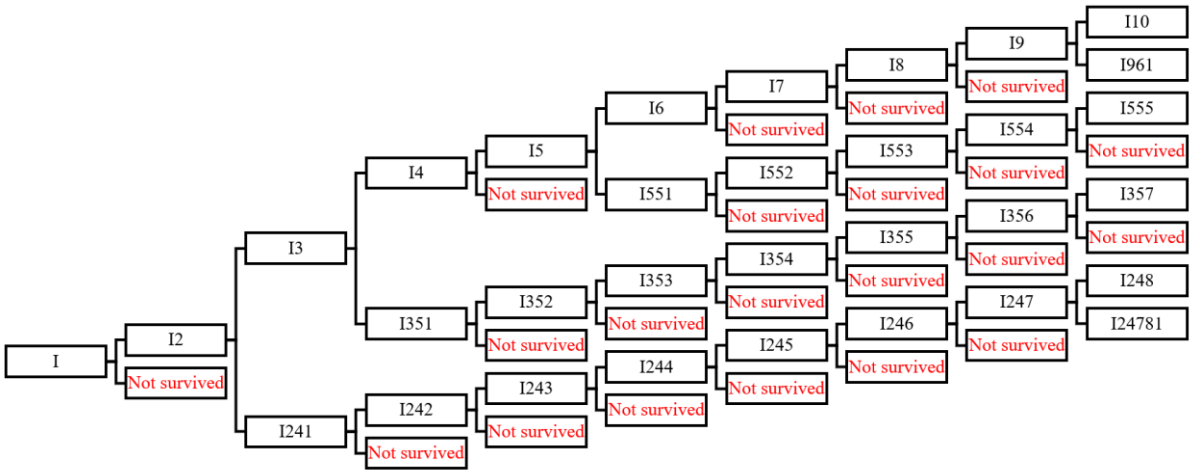


Figure 3. Optimization of initial cisplatin exposure for establishment of adaptive cisplatin-resistant HCT116 sublines. HCT116 cells were exposed to cisplatin for 2 treatment cycles. Distinct letters represent various cisplatin treatment conditions. HCT116 that were subjected to G1, G2, I and J survived and were utilized for subsequent establishment of adaptive cisplatin-resistant HCT116 sublines.

By the end of the experiment, out of a total of 86 flasks of cells, only 17 flasks of cells had survived cisplatin treatment (Figure 4). HCT116/G2A5B5 is the only cell line that survived pulsed cisplatin treatments (Figure 4A). 6 HCT116/I sublines had survived 10 passages with continuous cisplatin exposure (Figure 4B). Notably, highest number of HCT116 sublines survived treatments with lower initial cisplatin exposure dosage of 2 μM. 10 different HCT116/J sublines had survived at least 10 continuous passages in cisplatin-supplemented medium (Figure 4C). Notably, the HCT116/J10 subline was maintained in cisplatin-supplemented medium for three additional passages, resulting in the creation of the HCT116/J13 subline.



(A)



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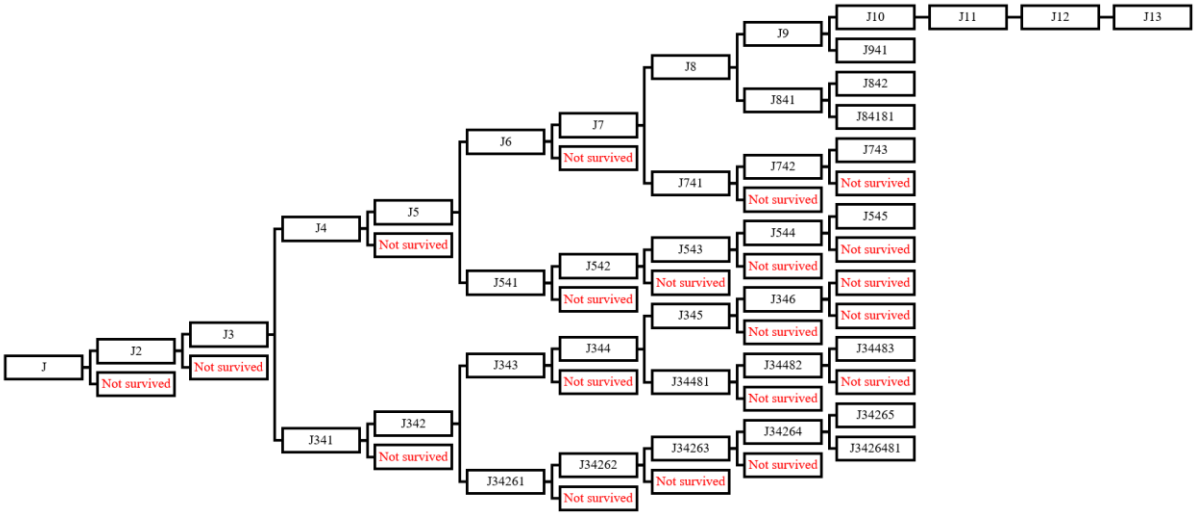


(C)

Figure 4. Cell lineage trees of the establishment of adaptive cisplatin-resistant HCT116 sublines employing different initial treatment strategies.

All 17 adaptive cisplatin-resistant HCT116 sublines exhibited significant greater tolerance to cisplatin treatment, as evidenced by their cisplatin IC_{50} values, which were significantly increased compared to parent HCT116 (Figure 5). The calculated acquired cisplatin-resistance strength ranged from 2.0- to 41.7-fold (Table 1). Among these 17 adaptive cisplatin-resistant HCT116 sublines, 6 displayed relatively strong acquired cisplatin resistance with cisplatin IC_{50} values exceeding 100 μM (Table 1). Notably, five out of six of the cell lines were established using continuous exposure method with stepwise dose increase. HCT116/J10, HCT116/J13 and HCT116/I10 that were established using continuous cisplatin exposure with constant dosing exhibited weak acquired cisplatin resistance of less than 5-fold increase in cisplatin resistance (Table 1). This finding demonstrates the success of employed method in selection of isogenic cell lines with acquired cisplatin resistance. Additionally, the finding also suggests that continuous drug exposure with stepwise dosage increase may be a better suited approach for the selection of isogenic cell lines with strong acquired drug resistance.

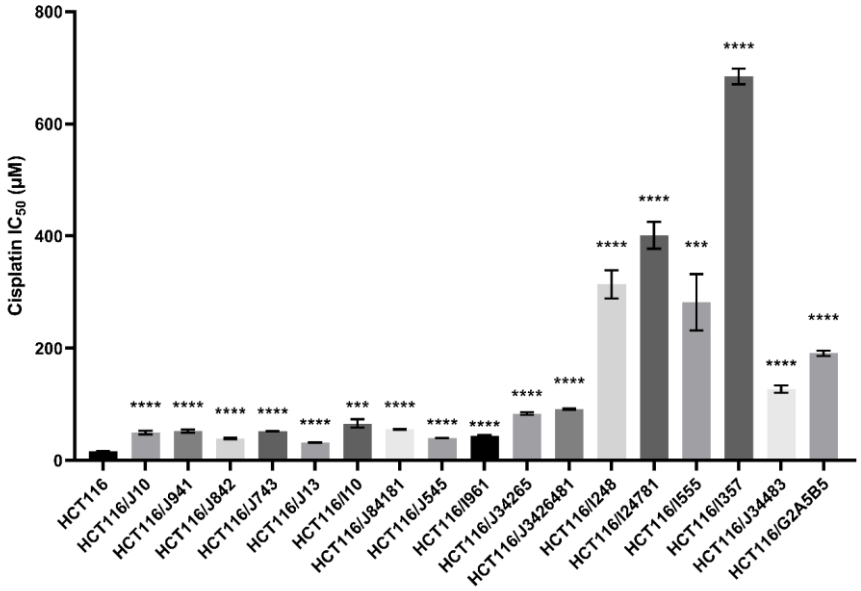


Figure 5. Comparison of cisplatin IC_{50} concentrations (24-hour treatment) in 17 adaptive cisplatin-resistant HCT116 sublines that survived the selection against parent cell line HCT116. Data represents mean ± standard deviation from three independent experiments (n=3). *** p<0.001; **** p<0.0001

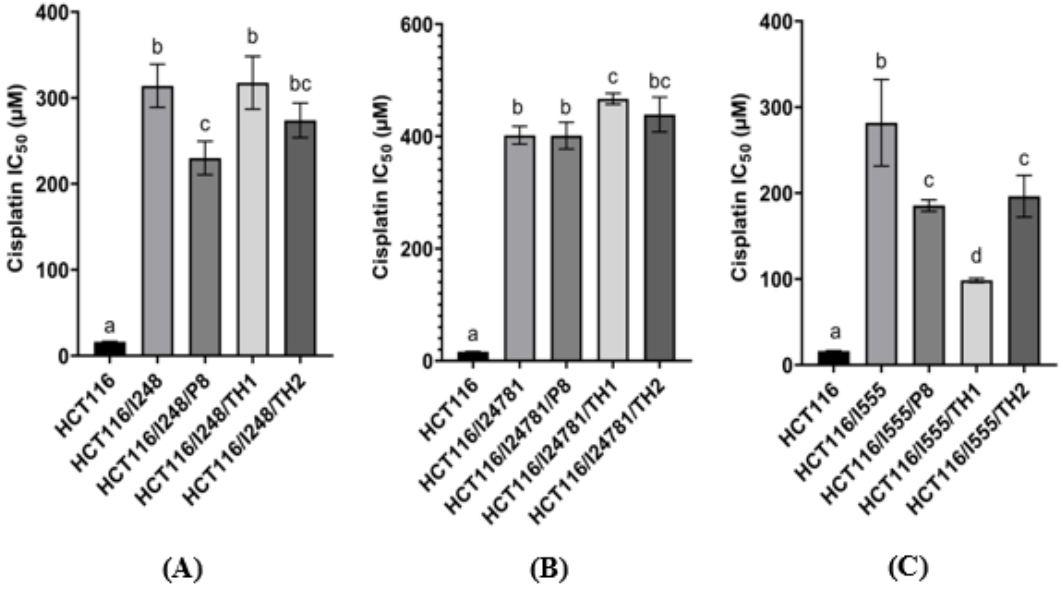
Table 1. Selection strategy of all 17 adaptive cisplatin-resistant HCT116 sublines and their respective cisplatin IC_{50} concentrations for 24-hour treatment. Fold-resistance indicates relative strength of acquired cisplatin-resistance.

Cell line	Exposure	Dosing	IC_{50} (cisplatin) (μM)	Fold-resistance (cisplatin)
HCT116	NA	NA	16.4	1.0
HCT116/J10	Continuous	Constant	49.7	3.0
HCT116/J941	Continuous	Stepwise	52.5	3.2
HCT116/J842	Continuous	Stepwise	39.1	2.4
HCT116/J743	Continuous	Stepwise	51.8	3.2
HCT116/J13	Continuous	Constant	32.1	2.0
HCT116/I10	Continuous	Constant	65.8	4.0
HCT116/J84181	Continuous	Stepwise	55.6	3.4
HCT116/J545	Continuous	Stepwise	39.5	2.4
HCT116/I961	Continuous	Stepwise	43.1	2.6
HCT116/J34265	Continuous	Stepwise	83.4	5.1
HCT116/J3426481	Continuous	Stepwise	91.3	5.6
HCT116/I248	Continuous	Stepwise	314.0	19.2
HCT116/I24781	Continuous	Stepwise	401.4	24.5
HCT116/I555	Continuous	Stepwise	281.8	17.2
HCT116/I357	Continuous	Stepwise	684.2	41.7
HCT116/J34483	Continuous	Stepwise	127.0	7.7
HCT116/G2A5B5	Pulsed	Stepwise	191.0	11.7

6 adaptive cisplatin-resistant HCT116 sublines with cisplatin IC_{50} exceeding 100 μM was assessed for the stability of their acquired cisplatin resistance. These cells were exposed to two different conditions to observe any changes in cisplatin resistance, as indicated by cisplatin IC_{50} values. The stability of an acquired drug resistance phenotype is considered confirmed if resistance persists when the cells are relived of the selective pressure for a prolonged period or subjected to another extreme stress.

Only HCT116/I24781 and HCT116/I248 exhibited relatively stable cisplatin resistance, where the IC_{50} of cisplatin did not decrease significantly after prolonged cisplatin withdrawal or after underwent two cryopreservation cycles (Figure 6). HCT116/I357 that developed the strongest cisplatin resistance quickly lost its resistance following cryopreservation stress (Figure 6D). The other three adaptive cisplatin-resistant HCT116 sublines, HCT116/I555, HCT116/J34483 and HCT116/G2A5B5 displayed a decline in acquired cisplatin resistance in either prolonged cisplatin withdrawal or cryopreserved (Figure 6C, 6E and 6F).

Together, in comparison to continuous drug exposure, cells established using pulsed drug exposure not only exhibited weaker resistance, the acquired cisplatin resistance was also not stable. Thus, this finding suggests that in contrast to pulsed drug exposure, continuous drug exposure strategy with stepwise dose increase is a better approach for the establishment of isogenic adaptive drug-resistant cell lines with strong and stable acquired resistance that is suitable for the study of chemoresistance mechanisms.



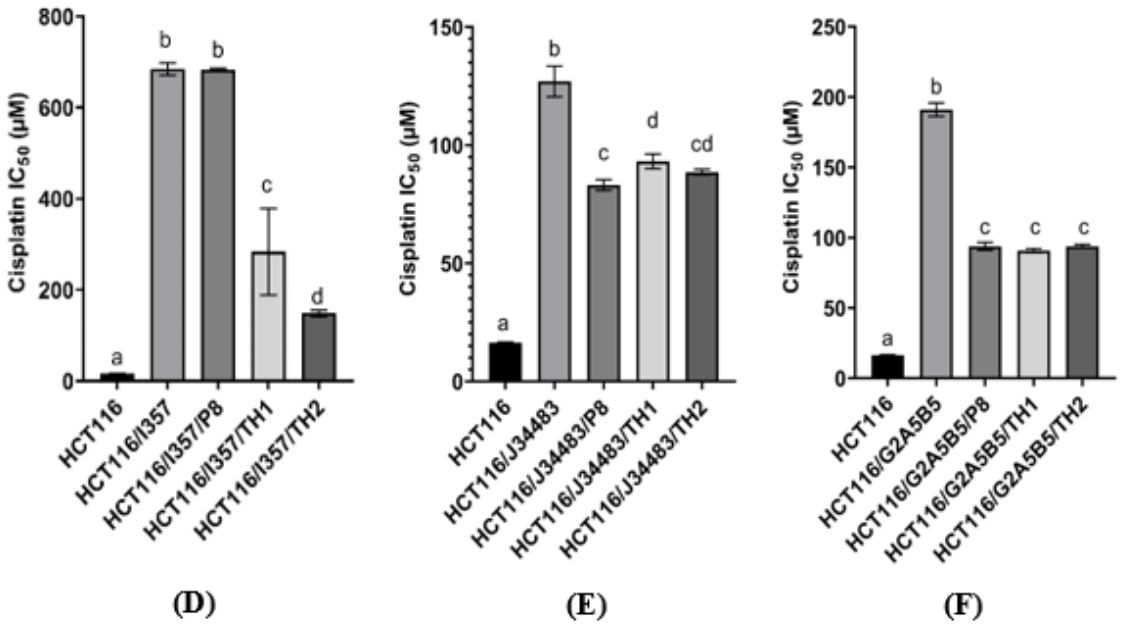


Figure 6. Resistance stability assay of six selected adaptive cisplatin-resistant HCT116 sublines. Changes in cisplatin IC_{50} were compared after resistant cells were alleviated off of cisplatin stress for prolonged period of time or subjected to freeze-thaw stress. (A) HCT116/I248 subline. (B) HCT116/I24781 subline. (C) HCT116/I555 subline. (D) HCT116/I357 subline. (E) HCT116/J34483 subline. (F) HCT116/G2A5B5 subline. Data represents mean ± standard deviation from three independent experiments (n=3). (P8 = 8 continuous passages in cisplatin-free medium; TH1 = 1 freeze-thaw cycle; TH2 = 2 freeze-thaw cycles).

All six HCT116 sublines that developed strong cisplatin resistance showed greatly reduced growth rate, with most noticeable difference in cell number observed at 72 hours post-seeding (Figure 7). Intriguingly, a discernible pattern emerged in the HCT116/I24781, HCT116/I248 and HCT116/I555 cells, wherein increased cisplatin resistance correlated with reduced growth rates (Table 2). The observations in this study are corroborated by other studies in which numerous published reports have described slower growth rate in cancer cells that developed a drug-resistant phenotype[13,14].

Table 2. Mean growth rate and doubling time of HCT116 and its adaptive cisplatin-resistant sublines, up to 72 hours post seeding.

Cell lines	Growth rate (cells/h)	Doubling time (h)
HCT116	0.0326 ± 0.0045	21.5 ± 2.9
HCT116/I24781	0.0202 ± 0.0071	37.8 ± 15.2
HCT116/I555	0.0254 ± 0.0069	29.7 ± 10.5
HCT116/I248	0.0237 ± 0.0064	31.0 ± 9.6
HCT116/I357	0.0162 ± 0.0133	272.7 ± 421.3
HCT116/J34483	0.0164 ± 0.0073	55.2 ± 36.8
HCT116/G2A5B5	0.0189 ± 0.0030	37.3 ± 6.3

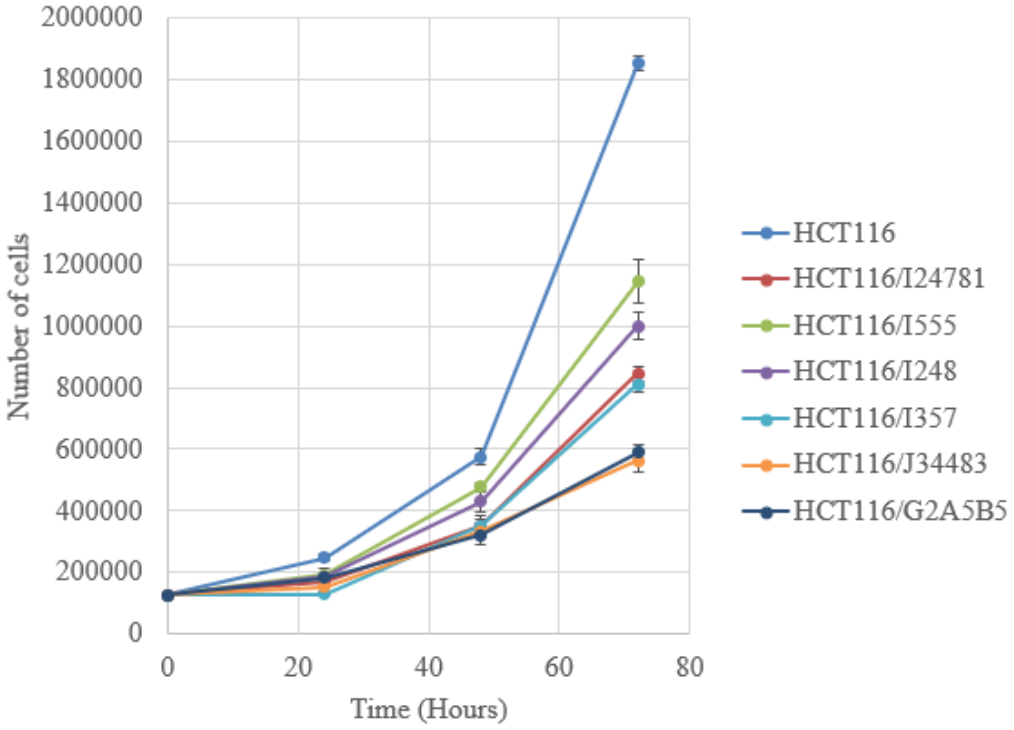
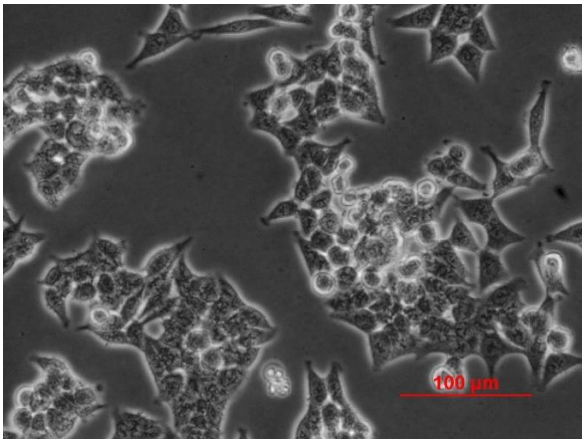
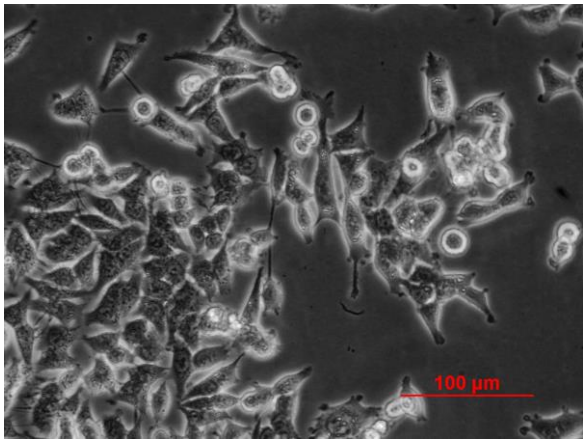


Figure 7. Growth curve of HCT116 and its adaptive cisplatin-resistant sublines. Cell number were determined at 24 hours, 48 hours and 72 hours post-seeding. Data represents mean ± standard deviation from three independent experiments (*n*=3).

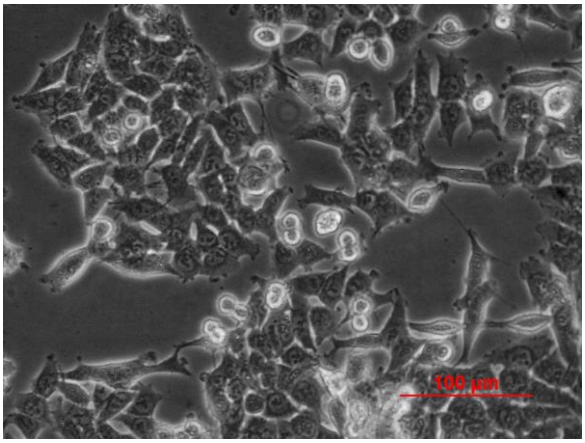
Phase contrast microscopy was performed on both HCT116 and its adaptive cisplatin-resistant sublines. The parental HCT116 cells exhibited an epithelial-like morphology (Figure 8A), which closely matched the documented characteristics provided by ATCC and corroborated by observations in other studies[15,16]. HCT116/I555 exhibited the closest similarity to the morphology of HCT116 (Figure 8D). While HCT116/I248 and HCT116/I24781 also closely resembled HCT116, subtle hints of arbitrary elongation were observed, with projection of cells becoming apparent (Figure 8B and 8C). Both HCT116/J34483 and HCT116/G2A5B5 displayed elongation of cells with a fibroblastic morphology, particularly noticeable in HCT116/J34483 (Figure 8F and 8G). Elongation of cells may play a role in mediating drug resistance in cancers, where elongation of breast cancer cells was reported to confer resistance to cisplatin[17]. In contrast, HCT116/I357 exhibited strong cell-cell adhesion, forming clumps of cells often with less visible cell borders (Figure 8E). Multiple studies indicated a correlation between increased cell adhesion and chemoresistance in cancer cells[18,19].



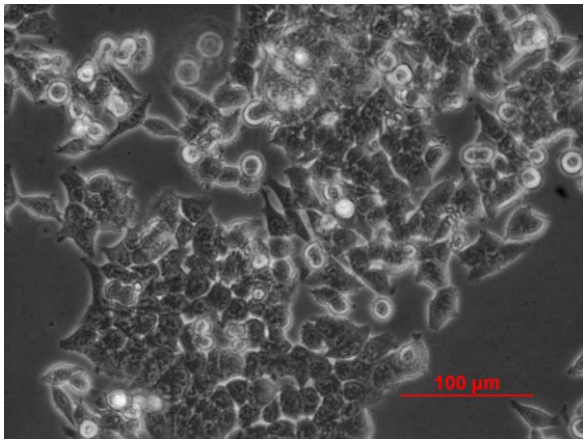
(A)



(B)



(C)



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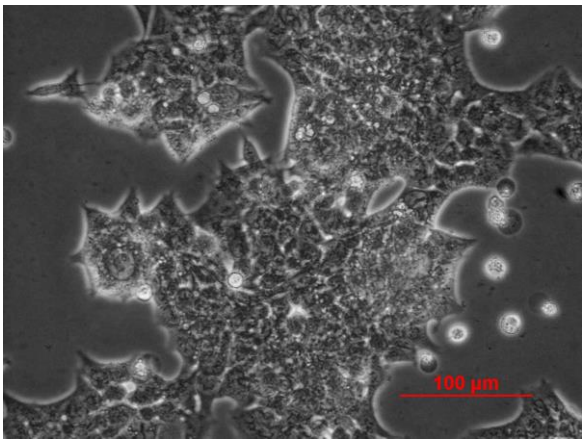

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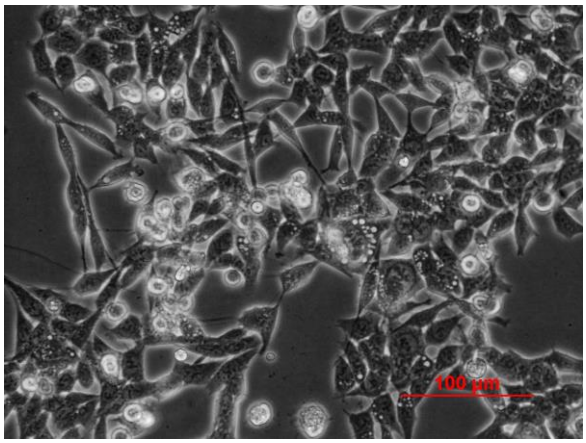
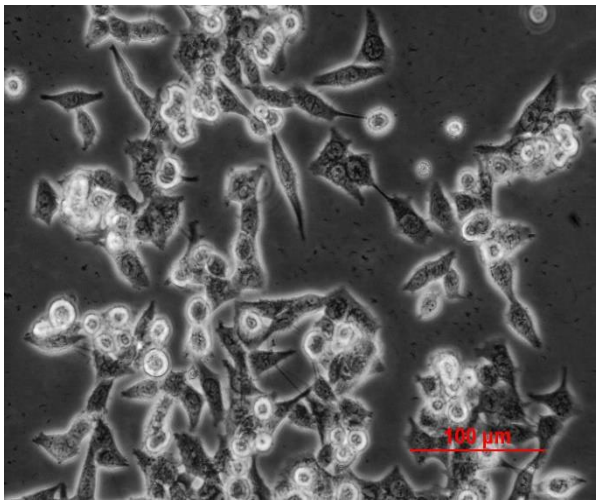


(F)



(G)

Figure 8. Phase-contrast microscopic images of HCT116 and its adaptive cisplatin-resistant sublines established in this study under 200x magnification. (A) HCT116 cells with epithelial-like morphology. (B) HCT116/I24781. (C) HCT116/I248. (D) HCT116/I555. (E) HCT116/I357. (F) HCT116/J34483. (G) HCT116/G2A5B5.

Scalebar represents 100 µM.

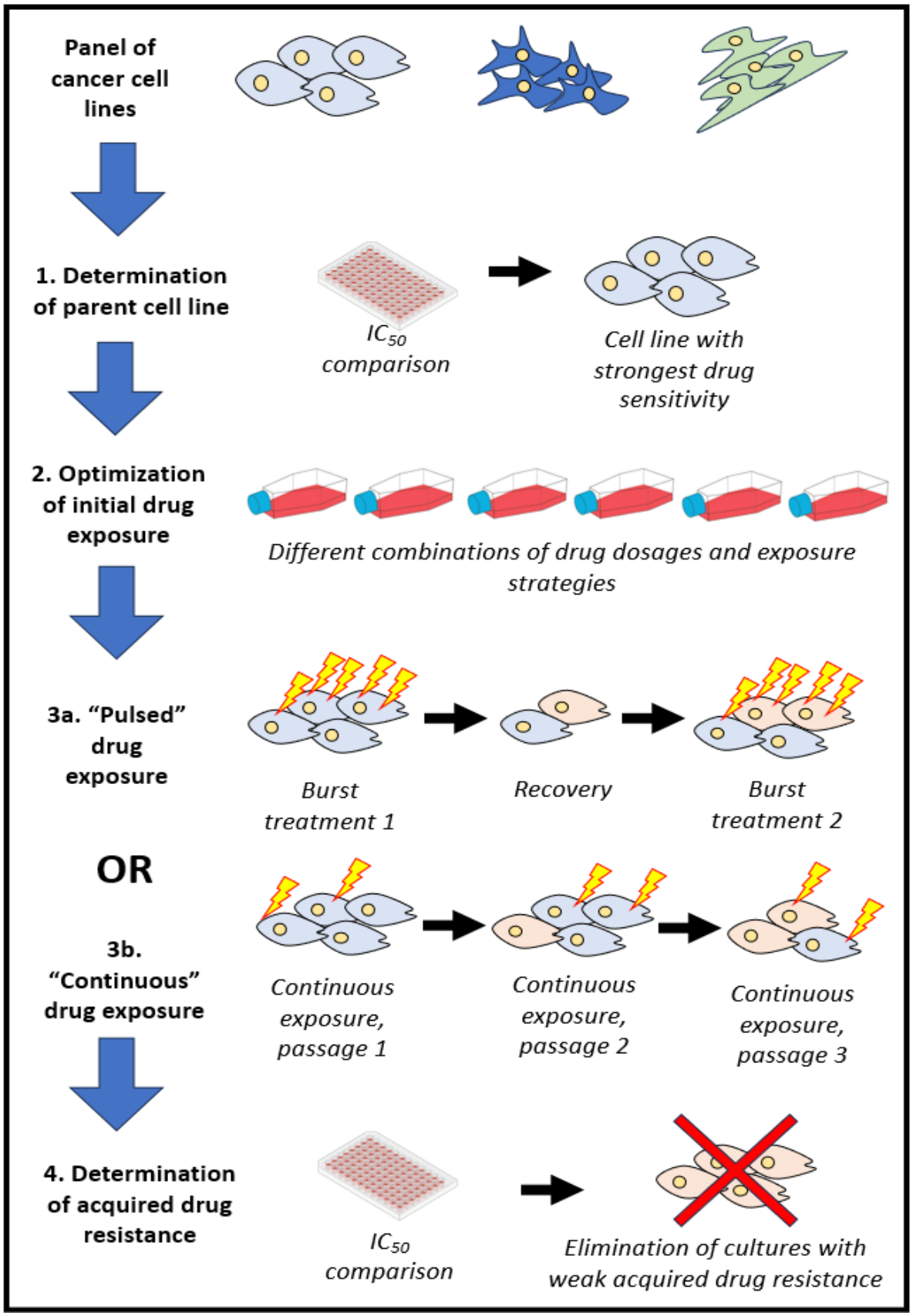
4. Conclusion

In summary, the methods outlined in this paper (Figure 1) facilitated the successful establishment of adaptive cisplatin-resistant HCT116 sublines (Figure 9). Notably, two of these sublines, HCT116/I24781 and HCT116/I248, exhibited stable acquired cisplatin resistance, rendering them suitable for chemoresistance studies. Importantly, these two HCT116 sublines were established using continuous cisplatin exposure with stepwise dose increase, implying that this method is suitable for the development of isogenic drug-resistant cell model for chemoresistance studies. Nonetheless, this paper summarizes the overall workflow of establishing isogenic adaptive drug-resistant cell lines, which involves:

1. **Selection of parental cell line:** Identifying a cell line exhibiting strong sensitivity to the drug of interest.
2. **Optimization of initial drug exposure:** Determining a suitable drug exposure strategy using the 24-hour IC_{50} concentration, of which should allow the parent cell line to survive for at least two treatment cycles.
3. **Conditioning of cells:** Employ a comparative selection strategy with a stepwise dose increase to enhance selective pressure while minimizing risk.
4. **Selecting cultures with adaptive drug resistance:** Determining the minimum number of treatment cycles the parent cells need to survive and assessing the strength of acquired drug resistance through IC_{50} comparison.

5. **Determination of resistance stability:** Assessing changes in the strength of acquired drug resistance in response to prolonged drug withdrawal and cryopreservation stress. This not only serves as evidence of resistance stability but also as a benchmark for handling adaptive drug-resistant cell lines to ensure the reliability of subsequent chemoresistance studies.
6. **Characterization:** Drug-resistant cancer cells typically exhibit a reduced growth rate. Additionally, although changes in cell morphology are not specific characteristics of cells with acquired drug resistance, they may provide insight into underlying chemoresistance mechanisms.

In conclusion, the methodology outlined in this study can serve as a valuable reference for researchers seeking to establish isogenic cell lines with acquired drug resistance. The evidence presented demonstrates the successful utilization of continuous drug exposure with a stepwise dose increase, resulting in the establishment of isogenic cell lines with robust and stable acquired drug resistance that are suitable for subsequent studies on chemoresistance. Importantly, the methods described are applicable not only to cisplatin resistance but also extend to other drug resistances and various cancer types beyond CRC.


Panel of cancer cell lines
1. Determination of parent cell line
IC_{50} comparison
Cell line with strongest drug sensitivity
2. Optimization of initial drug exposure
Different combinations of drug dosages and exposure strategies
3a. "Pulsed" drug exposure
Burst treatment 1
Recovery
Burst treatment 2
OR
3b. "Continuous" drug exposure
Continuous exposure, passage 1
Continuous exposure, passage 2
Continuous exposure, passage 3
4. Determination of acquired drug resistance
IC_{50} comparison
Elimination of cultures with weak acquired drug resistance

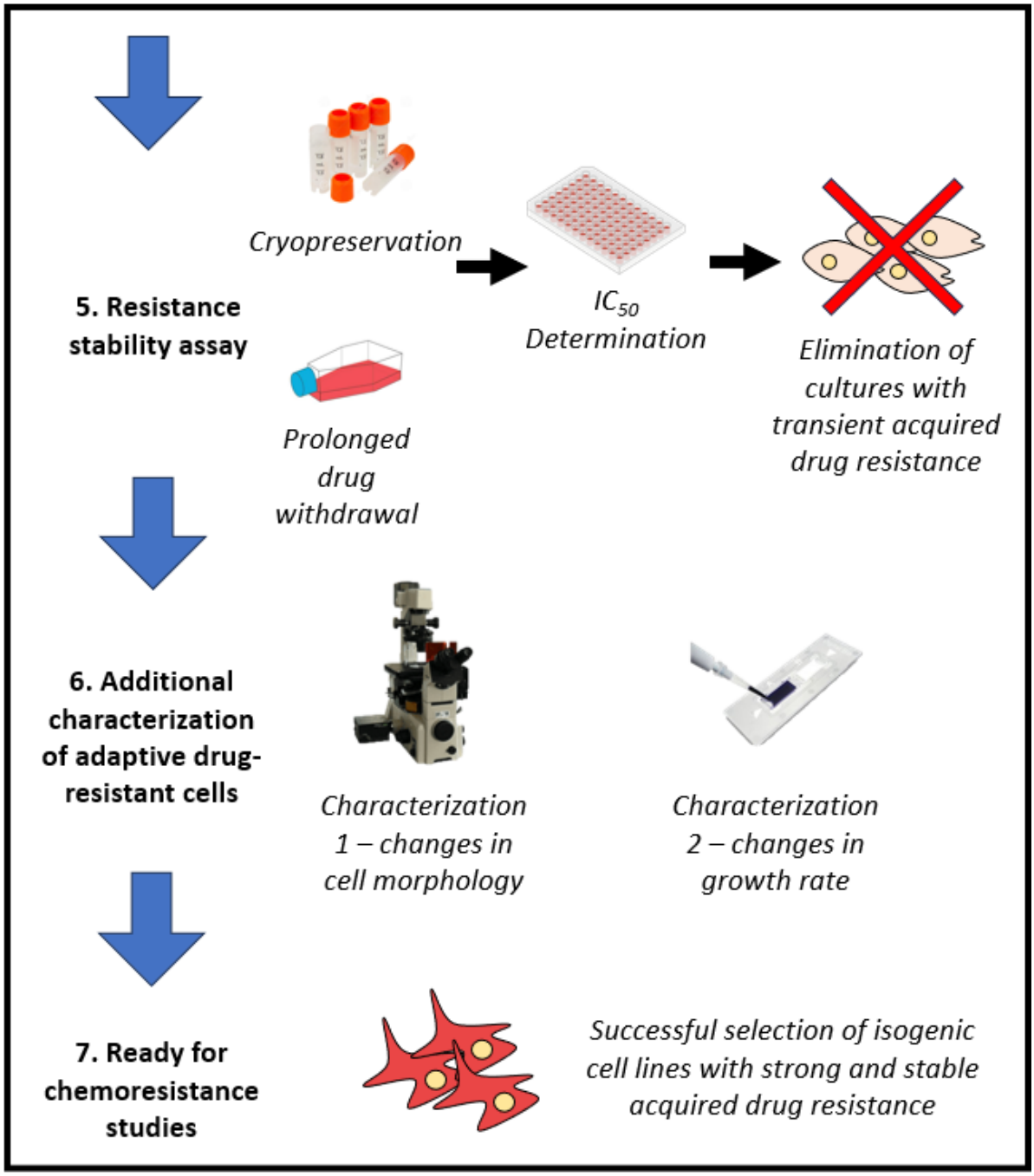


Figure 9. Graphical summary of establishment of adaptive cisplatin-resistant CRC cell model for chemoresistance studies.

Author Contribution: ZF, BHG and WL researched literature and conceived the study. ZF was involved in protocol development, data collection and data analysis. ZF wrote the first draft of the manuscript. All authors reviewed and edited the manuscript 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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