

**ORIGINAL RESEARCH ARTICLE** 



# Targeting liver cancer stem cell through EpCAM therapy targeted with chemotherapy endorse enhanced progression in hepatocellular carcinoma



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# Abstract

**Background** Two chief hurdles in most cancer treatments are chemoresistance and tumor recurrence, especially counting hepatocellular carcinoma (HCC). Most conformist chemotherapy fails to completely cure HCC patients because of its susceptibility to develop multidrug resistance (MDR) through factors such as hypoxia, cancer stem cells, and drug efflux mechanism cancer stem cells (CSC) which are significant factors involved in chemoresistance. It has been exposed that targeting liver cancer stem cells and chemotherapeutic drugs have a better selected, overall survival rate for hepatocellular carcinoma patients.

**Aim** This study aims to investigate the effectiveness of targeting stem cells for liver cancer using a therapy that targets EpCAM in combination with chemotherapy and how this approach can enhance the treatment outcomes in hepatocellular carcinoma, the most prevalent kind of liver cancer.

**Results** The outcome was studied by flow cytometry, Western blot, RT-PCR, and cytotoxicity assays. EpCAM gene silenced and XAV939-treated cells showed decreased expression of CD133, a liver cancer stem cell (LCSC) marker in flow cytometry analysis, and reduced expression of ABCG2 gene, which is a reliable marker for chemoresistance in RT-PCR and western blot analysis; it was also unable to form colonies in colony forming assay. Similarly, in the spheroid formation assay, EpCAM gene silenced cells and XAV939-treated cells in combinations with cisplatin treatment were powerless to appear spheroid, whereas cisplatin alone-treated cells showed spheroids. In the cytotoxicity assay, cisplatin alone and combined with EpCAM silenced and XAV939-treated cells showed more lactate dehydrogenase (LDH) release than EpCAM silenced arm XAV939 treated components.

**Conclusion** These findings confirm our hypothesis that conventional chemotherapy kills cancer cells but not cancer stem cells. We believe EpCAM-targeted therapy enhances chemosensitivity and decreases relapsed chances. This approach might be the best option for a better prognosis for hepatocellular carcinoma patients.

Keywords Cancer stem cell, EpCAM, Hepatocellular, Wnt- $\beta$  catenin, XAV939

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# Introduction

The liver is a vital organ current in vertebrates. It plays a chief role with more than 400 functions, including producing plasma protein, controlling homeostasis, glycogen, lipids storage, albumin, bilirubin production, detoxification of xenobiotics, and vital roles in metabolism. Hepatocellular carcinoma (HCC) is the world's third-largest cause of cancer mortality and the sixth most widespread cancer [1]. Cirrhosis of the liver is a significant health issue in India as well. According to the most recent WHO data from 2017, 259,749 individuals died from liver disease in India, accounting for 2.95% of all fatalities and 18.3% of all cirrhosis deaths globally [2].

The most crucial etiologic cause of HCC is chronic viral infections such as hepatitis B and C, similar to metabolic disorders, and chronic alcoholism is also a modest concern in HCC [3]. Surgical treatment, radiotherapy, and chemotherapy are the standard management options for HCC. Sorafenib is the common food and drug administration (FDA) accepted drug for HCC. Majority of the conservative treatments, such as radiotherapy and chemotherapy, fail to treat HCC patients because of numerous factors, including tumor microenvironment, DNA damage repair, ATP overexpression, compulsory cartridge drug efflux [4, 5], the hypoxia-epithelial-mesenchymal transition (E.M.T.), inducible factor  $1-\alpha$  (Hif $1-\alpha$ ) [6] calcium signaling, autophagy induction [7, 8], cancer stem cell, epigenetic regulation [9, 10], miRNAs, and immunosuppressive microenvironment [11, 12]; moreover, many of these facilitate resistance in multidrug use. Among all the above factors, CSC (tumor-initiating cells) plays a significant role in self-renewal, metastasis, chemoresistance, and radioresistance, leading to a high chance of tumor relapse. Since the concept of CSCs came into prominence in the belatedly 1990s, it has regularly gained universal reception and prejudiced all approaches to cancer investigation and therapy [13]. Researchers have found that chemotherapy kills cancer cells but does not affect the cancer stem cells. A minimum viable portion within the tumor, cancer stem cells, show chemoresistance features to some chemotherapy. Dallas et al. [14] showed that 5-fluorouracil and oxaliplatin drugs show chemoresistance to the colorectal cancer cells and enhance the CSC population almost 5-22-fold. Hermann et al. [15] claimed that human pancreatic cells show chemoresistance to the gemcitabine treatment and a 50-fold increase of cancer stem cell marker CD133 expression. Our previous research demonstrated that cisplatin drugs ultimately face difficulties in killing Huh7 cells. These cisplatin-resistant cells showed higher expression of liver cancer stem cell markers such as CD133 and EpCAM. In addition, these cells strongly expressed stem cell transduction factors like Oct4 and Nanog.

Similarly, only cisplatin-resistant cells formed a spheroid, not the Huh7 cells. These findings confirm that Huh7 cells are resistant to cisplatin drug, and those resistant cells express cancer stem cell-like behaviors such as chemoresistance and tumor recurrence [16]. EpCAM was initially identified as an epithelial marker; consequently, researchers found it is also a cancer stem cell marker for several epithelial cancers. EpCAM is involved in various tumor processes such as proliferation, invasion, tumorigenicity, and metastasis, mainly through the wnt- $\beta$ -catenin pathway and other signaling pathways [17]. So, the combinatorial approaches targeting cancer stem cells and cancer cells could be the right choice for tumor-free survival.

# **Materials and method**

# **Chemicals and reagents**

NCCS (National Centre for Cell Science) provides cancer cells, so the cell lines Huh7 were purchased from NCCS, Pune, India. Antibiotic–Antimycotic (cat#15,240,062) and Fetal bovine serum (Cat#11,573,397) was purchased from Gibco (Cat#41,400,045) ITS from Invitrogen. Cisplatin (Cat#1550) from Bio vision, XAV939 from Selleckchem, ABCG2 (Cat#ab3380) from Abcam, FITC-conjugated CD133 monoclonal antibody (Cat#11–1339-41, clone #EMK08) eBioscience, PE-conjugated EpCAM monoclonal antibody (clone#EBA-1, Cat#347,198), and shRNA (EpCAM) kit from Gibco.

### The generation of LCSC-enriched Huh7 cell line variants

Huh7 Cells were cultured with a low dose of 1.655 µg/ml of cisplatin in absolute RPMI 1640 medium supplemented with F.B.S. (10%), ITS, antimicrobial for 37 °C (5 days) in CO<sub>2</sub> (5%) Incubator, and the medium was distorted every 2 days to remove the dead cells. Cisplatin was supplementary while the logarithmic enlargement phase was reached. The generation of chemo-resistant cell variants of HuH7 cells is established through the next to final confrontation to cell death through the treatment with cisplatin. The consequential phenotype resistance was confirmed constant over  $\geq 6$  passages devoid of cisplatin [18].

### **Cell culture**

LCSC-enriched HuH7 cell line variants were cultured in complete RPMI 1640 medium with F.B.S. (10%), ITS, and antimicrobial up to three passages to obtain sufficient cells, after these cells were seeded in six-well plates and incubated at least for 24 h; previously reached exceeding  $\sim$  70% convergence. These cells were cultured in

six different groups, such as XAV939 (13.4 nM) alone, EpCAM gene silence using shRNA alone, cisplatin alone, XAV939 combined with cisplatin, EpCAM knockdown with cisplatin, and untreated cells as a control for 3 days at 37 oC in CO2(5%) incubator. Then these cells were harvested for subsequence analysis [18].

### Silencing EpCAM gene using shRNA

RNAi mediate thump over was performed with the subsequent diminutive meddlesome RNA (shRNA): EpCAM-1: 59-UGCUCUGAGCGAGUGAGAATT-39; EpCAM-2:59 UUCUCACUCGCUCAGAGCATT-39, unconstructive control shRNA, was used in every experiment as a non-silencing control shRNA. All shRNAs (20 nM) target EpCAM was established in cells using lipofectamine 2000 reagent per the manufacturer's procedures [19].

# Flow cytometry

FITC conjugated CD133 and APC conjugated EpCAM PE-conjugated was purchased in BD Bioscience. After 3 days, XXAV939- and shRNA (EpCAM)-treated LCSC-enriched huh7 cell variants were dissociated with trypsin–EDTA (0.25%;1 mM) (Invitrogen) for 3 min and washed with Ca and Mg free Dulbecco PBS solution through spinning at 400 g for 7 min. Then these cells were watered down in FACS buffer (100  $\mu$ l; PBS contains fetal calf serum 1%) and then incubated for 1 h at 4 °C in FACS buffer with the equivalent mAb: anti-CD133-FITC and anti-EpCAM-APC. The flow cytometry examination was performed with a BD FACS Canto II flow cytometer (BD Biosciences) [20].

### **RT-PCR**

The total RNA has been removed with the TRIzol (Invitrogen) reagent from all the drug-treated cell groups, including the control. Primary thread flattering DNA (cDNA) was manufactured, starting entirety RNA according to the manufacturer's procedure for RNA PCR kit (Madison, Promega, WI, USA). PCR quantitatively was carried out using the ABI7300 RT-PCR system (Applied Biosystems, CA). ABCG2 appearance was enumerated, and  $\beta$ -actin was used as an endogenous orientation. Results were uttered as go bankrupt change in gene expression [20].

### Western blot

The reagents extracted nuclear and cytoplasmic proteins; nuclear and cytoplasmic extraction (Pierce). Proteins were electrophoresed on SDS-PAGE gels and transferred to the nitrocellulose membrane (Invitrogen). Protein detection was completed using the following antibodies: anti-ABCG2 monoclonal antibody (R&D Systems, Inc.) and anti- $\beta$ -actin monoclonal antibody (Sigma). Bounce antibodies were detected by chemiluminescence detection-enhanced reagents (Amersham Biosciences) [20].

### Spheroid formation assay

Liver cancer stem cells enriched Huh7 cell line variants were treated with XAV939 alone, shRNA (EpCAM) alone, cisplatin alone, XAV939 combination with cisplatin, shRNA (EpCAM) combination with cisplatin, and DMSO alone as a control in several 1000 per 0.2 ml of complete stem cell medium in 96-well plates. 3D cell culture reagent Matrigel (Cat. No: 354230BD Biosciences) was used to civilization liver spheroids. The 5 mg/ml attentiveness of matrigel was equipped and used for culture spheroids. Incubate the cells under paradigm conditions at  $37^{\circ}$ C and CO2 (5%) for the most favorable spheroid size for 2 weeks. A rigid margin spheroid was observed by day 9. Culture media is replenished with freshly prepared complete stem cell medium every 2–3 days up to 9 days [20].

### **Colony forming assay**

The colony or clonogenic configuration is an assay of in vitro cell endurance based on the capability of a solitary cell to produce a dependency. The dependency is distinct from consisting of at least fifty cells. After management, cells are seeded out in suitable dilutions to appear colonies in weeks 1–3. Colonies are permanent with glutaraldehyde (6.0%; v/v), discolored with crystal violet (0.5%; w/v), and calculated by a stereomicroscope [20].

### Viability

The Huh7 cell line was grown in 96-well plates with drugs and appropriate controls. Consequently, they were incubated with reagent WST-1 (DojindoInc, Japan) for 4 h. Formosan dye fashioned was quantified with a multi-well spectrophotometer (ELISA reader). The calculated straight absorbance compared to the no of viable cells [21].

### Cytotoxicity

The Huh7 cell line was grown in 96-well plates with controls and suitable standard drugs, and the supernatant was collected and incubated with LDH reagent (Caymen) for 30 min. After this incubation period, the amount of LDH release was quantified with a multi-well spectrophotometer (ELISA reader), and the absorbance measured unswervingly correlated to the no of dead cells [21].

### Statistical analysis

The two-tailed Student *T*-test and GraphPad Prism software will be used for the statistical analysis. Results with less than a 5% chance of occurrence will be considered statistically significant since the significance level will be fixed at p < 0.05. Under the assumptions of a normal distribution and equal variance, the two-tailed Student *T*-test is frequently used to compare the means of two groups of continuous data. The test determines the *t*-value, the distinction between the two means adjusted by the difference's standard error. A successful show for scientific data analysis and graphing is GraphPad Prism. It offers an easy-to-use interface that enables users to run different statistical tests and create graphs that are suitable for study.

# Results

# Absence of tumor recurrence properties in EpCAM-targeted cells

EpCAM plays a vital role in cancer stem cell activation, tumor invasion, and metastasis. EpCAM gene was silenced in LCSC-enriched Huh7 cell lines variants through shRNA technology. Further, to evaluate the tumor-initiating properties of EpCAM, these EpCAM knockout cells were cultured for colony-forming assay along with untreated cells as a control. Interestingly EpCAM knockdown cells were unable to form colonies in colony forming assay, whereas control cells were able to form colonies. It has indicated that EpCAM-positive cells are tumor-initiating cells (Fig. 1).

The wnt- $\beta$ -catenin signaling passageway is an explanation regulating pathway for cell proliferation, invasion, metastasis, and maintaining stemness. XAV939, a tankyrase 1 inhibitor, promotes cell apoptosis by inhibiting the wnt- $\beta$ -catenin signaling pathway. LCSC-enriched cells alone as a control, and XAV939 treated cells were cultured for the colony-forming assay. This assay helps to identify the functional assessment of tumor recurrence properties. Fortunately, XAV939-treated cells were unable to form any colonies. This data confirms that wnt- $\beta$ -catenin signaling is essential in the signaling pathway for cancer stem cell activation and tumorigenesis, and EpCAM-positive cells are the tumor-initiating cells (Fig. 1).

# Evaluation of chemoresistant property in EpCAM-targeted therapy

To evaluate the efficacy of cisplatin-sensitive on XAV939 (wnt  $\beta$ -catenin inhibitor) and EpCAM gene silenced by shRNA on LCSC-enriched Huh7 cell variants, these treated cells were studied ATP binding cassette G2 (ABCG2) gene, which is highly expressed in many chemo-resistant cancers. The expression of ABCG2 is directly correlated with resistance to chemo drugs. ABCG2 gene expression was analyzed by RT-PCR and western blot analysis in cisplatin-treated (+ve control), ShEpCAM-treated, and XAV939-treated cells. The results observed in RT-PCR analysis showed a significant decrease of ABCG2 gene expression in sh-EpCAM- and XAV939-treated cells, whereas in cisplatin-treated cells, the aberrant expression of ABCG2 was observed. Correspondingly in western blot analysis, ABCG2 expression was drastically decreased in sh-EpCAM- and XAV939treated cells. These findings support our hypothesis that sh-EpCAM and XAV939 treatment suppresses the expression of ABCG2, and a combination of cisplatin along with sh-EpCAM, or XAV939, might help for better progression in EpCAM-positive hepatocellular carcinoma (Fig. 2a, b).

# Reduced expression of CSC-associated gene after EpCAM-targeted therapy

CD133 and EpCAM are well-known markers for liver cancer stem cells by numerous researchers. Flow



Fig. 1 shRNA(EpCAM) silenced cells and XAV939-treated cells were not able to form any colony as appeared in the control arm. a Control. b shRNA(EpCAM). c Wnt targetted



Fig. 2 a ABCG2 expression was drastically decreased in both EpCAM-targeted therapy compared than in cisplatin-treated cells. **b** ABCG2 a known chemoresistant marker was significantly decreased in XAV939-treated cells compared to the positive control



Fig. 3 Liver cancer stem cell expression was drastically decreased in EpCAM-targeted therapy in single or in combination compared to control and + ve control (cisplatin treatment)

cytometry analysis was carried out to evaluate the expression of LCSC in sh-EpCAM, XAV939, cisplatin alone, cisplatin with sh-EpCAM, and cisplatin with XAV939-treated LCSC-enriched Huh7 cell variants. The results showed an aberrant decrease of EpCAM and CD133 expression in single and combination with EpCAM-targeted therapy, whereas in control, the expression remained the same, and in cisplatin-treated arms, the LCSC expression was drastically elevated. These results confirm that sh-EPCAM, XAV939 as a single, or combination therapy might suppress the LCSC activation drastically in the Huh7 cell line (Fig. 3).

# Inhibiting the tumor recurrence properties in a combination of chemotherapy along with EpCAM-targeted therapy

Chemoresistance and tumor deterioration are unfortunate but prevalent impediments in cancer therapy. EpCAM-targeted therapies such as shRNA (EpCAM) and XAV939 inhibit tumor initiation, colony formation, tumor invasion, and decreased expression of the chemo-resistant gene ABCG2. CSC-enriched Huh7 cells were cultured with XAV939, shEpCAM, cisplatin individual, and a combination of cisplatin with XAV939 and cisplatin with shEpCAM for spheroid formation assy. Spheroid formation assay, a novel in vitro assay, helps to study the efficacy of inhibiting tumor formation by EpCAM-targeted therapy as an individual or in combination with cisplatin. Cells without any drugs were cultured as a control. Interestingly none of the EpCAM-targeted therapy individuals and the combination with cisplatin could not form a single colony, whereas cisplatin alone and control cells show spheroids after the 9th day of culture. These findings confirm that the combinatorial approach of EpCAMtargeted therapy alongside cisplatin improves progression in EpCAM-positive hepatocellular carcinoma (Fig. 4).

# EpCAM-targeted therapy promotes chemo-sensitivity to cisplatin

CSC-enriched cells were cultured without drug as a control and with cisplatin alone, XAV939 alone, shEpCAM alone, XAV939 with cisplatin, and shEpCAM with cisplatin for 3 days. Drug media were refreshed every 24 h. The culture supernatant was collected at all the time points for cytotoxicity assay in kinetic. After the third day of culture, the cell viability was measured using M.T.T. XAV939 and sh-EpCAM treatment arms noted a slight drop in viability compared to the control. In cisplatin alone, combined with sh-EpCAM and with XAV939 treated cells, viability was drastically decreased than control. The cytotoxicity assay observed an elevated LDH expression in cisplatin alone and combined with XAV939 and shEpCAM treatment than control. However, in treating XAV939 alone and sh-EpCAM alone, cells released much lesser LDH than in the combination. This data suggests that EpCAM-targeted therapies and cisplatin promote chemo-sensitivity in liver cancer (Fig. 5a, b).

# Discussion

Chemoresistance and tumor recurrence is the major obstacle in cancer therapy. To understand the molecular mechanism of tumor recurrence, we should know the molecular difference between primary and relapsed tumors. Adam et al. and his group reported that the primary tumor showed low expression of CD44, ALDH1A1, and CD133, whereas the recurrence tumor after the



Fig. 4 Spheroid formation assay—EpCAM-targeted therapy either in single or in combination inhibit the cells which responsible for form spheroids. a Control. b Cisplatin. c XAV9393. d. XAV3939 + cisplatin. e shRNA (EpCAM). f shRNA (EpCAM) + cisplatin



**Fig. 5** a In cytotoxicity assay, cisplatin alone and combination with XAV939 and (EpCAM)-treated cells shown higher cytotoxicity level than in XAV939 and shRNA (EpCAM) alone-treated cells. b Similarly, in cell viability assay, cisplatin alone and combination with EpCAM-targeted therapies shown decreased viability compared to the XAV939 alone and shRNA (EpCAM) alone treatments

chemotherapy showed an increased expression of CD44, ALDH1A1, and CD133. CD133 expression was significantly increased by about 14% in platinum-resistant patients compared to the matched most important tumors [22]. Tsunaki Yamashina et al. [23] and colleagues found that cancer stem-like cells develop chemo-resistant tumors by producing an assortment of pro-inflammatory cytokines and generating M2-like immunoregulatory myeloid cells from CD14+monocytes [23]. FundaMeric-Bernstamet al. [24] and his team reported that by understanding the genomic alteration between the most critical and persistent breast tumors, they observed the increased frequency of CDK4/MDM2 amplifications in recurrences than the primary tumor.

Cheng-Wei Lin et al. [25] demonstrated how EpCAM plays a significant role in the tumor; they have found that EpCAM and stem cell transcription factors (Sox2, Nanog, Oct4, and c-Myc) be simultaneously prominent in TICs, which lead to more personality regeneration, invasive-ness, enhanced tumorsphere, and tumor initiate ability. In further, EpCAM knockdown inhibited the reprogramming expressions feature and epithelial-mesenchymal

transition (EMT) genes, in so doing tumor suppress initiation, invasiveness, and self-renewal [25]. According to their research, Yi Chen et al. [26] concludes that EpCAM+CD133+cells have the characteristic of tumor initiate cells compared to CD133-EpCAM+, CD133+EpCAM-, and CD133-EpCAM-cells. In addition to that, CD133+EpCAM+cells showed elevated differentiation capability, increased formation capability, higher expression of stem cell transduction factors, drug-resistant to some chemo-drugs, better spheroid formation and spheroid solid formation, and strong tumorigenicity in NOD/SCID mice [26].

The signaling of Wnt/ β-catenin necessary pathway for upholding stemness in normal livers and liver carcinomas, particularly in Wnt/β-catenin signaling HCC, acting as a chief responsibility in the spheroid configuration as well as the continuation of acinous. Atsushi Takai et al. [27] reported that positive EpCAM cells are supplementary responsive for TGF/ $\beta$  encouraged epithelialmesenchymal transition with extremely metastatic and tumorigenic impending in vivo. Curtis et al. [28] and his colleagues confirmed through flow cytometry that wnt-βcatenin signaling embarrassment could decrease EpCAM expression, wnt-β-catenin signaling pathway inhibitor (XAV939), and EpCAM gene silencing through shRNA inhibited the liver cancer stem cells expression. Grippingly in the colony formation assay, there was no colony formation in XAV939-treated cells compared to the control. So, sh-EpCAM and XAV939 treatment inhibits tumor initiation and invasion properties by suppressing the cancer stem cell.

Partha Krishnamurthy et al. [29] and his colleague reveal that the marker and A.B.C. transporter progenitor and stem cells are recognized as the breast cancer resistance protein (ABCG2 or BCRP), conferring a brawny survival advantage under hypoxic conditions. Guang Zhang et al. [30] and Wu et al. [31] and his team studied the characteristic of ABCG2-positive cells in HCC. They found that ABCG2-positive cells expressed high tumorigenicity, enhanced cell proliferation, improved migration, and were doxorubicin-resistant, whereas, in ABCG2, downregulation led to inhibition of tumorigenicity, suppressed tumor invasion, and sensitivity to doxorubicin chemo drugs [32, 33].

Our data demonstrate the aberrant wnt- $\beta$ -catenin activation in LCSC-enriched Huh7 cell variants. The aberrant wnt- $\beta$ -catenin activation was suppressed by targeting XAV939 and enhanced the cisplatin sensitivity. To support this data, ABCG2 expression was drastically decreased in both shEpCAM- and XAV939-treated cells, but in contrast, the expression was increased in cisplatin-treated LCSC-enriched Huh7 cell variants. The viability of XAV939 and sh-EpCAM treatment arms was slightly

dropped compared to the control, but cisplatin alone and combined with sh-EpCAM and with XAV939 treated cells viability drastically decreased than the control. The cytotoxicity assay observed an elevated LDH expression in cisplatin alone and combined with XAV939 and shEp-CAM treatment than control. However, in XAV939 alone and shEpCAM alone, treatment cells have much lesser LDH release than the combination. Interestingly, in the spheroid formation assay, none of the EpCAM-targeted therapy, neither individual nor combination with cisplatin, could form a spheroid, whereas, in cisplatin alone and control, cells showed spheroids. Due to the study's limitations, the drug dose can only be assessed indirectly through gene expression, which can substitute for XAV939 or EpCAM knockdowns. In addition to cellular absorption, drug metabolism, and protein stability, other factors may be affecting knockdown effectiveness. Even though conventional therapies may have some promising results against tumors, they are fraught with limitations that result in local recurrences, metastases, and poor long-term survival. Alternative treatment approaches for liver cancer may target CSCs instead of conventional therapies.

### Conclusion

EpCAM-targeted therapy such as sh-EpCAM and XAV939 were proven to be potential inhibitors of liver cancer stem cells by inhibiting colony formation, invasion properties, and decreased expression of ABCG2, the gene responsible for chemoresistance. The LCSC expression and spheroid formation were drastically reduced in single and combination cisplatin, and EpCAM-targeted therapy. Overall, targeting EpCAM with therapies such as sh-EpCAM and XAV939 shows promise as a potential treatment for liver cancer, although further exploration is needed to regulate their efficacy and safety in clinical trials. These studies imply that combining cisplatin and EpCAM-targeted therapy may benefit both and improve cancer treatment outcomes. More research is required to determine the ideal dose, schedule, and patient selection for this combination therapy approach. It would be necessary to conduct further research to determine whether combining XAV939, EpCAM knockdown, and cisplatin could synergistically affect stem cell populations. A well-designed controlled study with appropriate sample size, randomisation, and statistical analysis is typically required to determine whether the combination of cisplatin and a specific drug has a synergistic, additive, or antagonistic effect. Research should be conducted to validate any claims regarding drug interactions to ensure that the results are accurate and reliable. These assays may provide functional evidence of the effects of the treatments on stem cell properties.

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### Authors' contributions

VS has written the contents. RV and AP corrected the data and edited the figures. GS designed the study, corrected, revised, and approved the manuscript for submission. All the authors approved the manuscript for publication.

### Availability of data and materials

Applicable.

### Declarations

**Ethics approval and consent to participate** Not applicable.

#### **Consent for publication**

Not applicable.

#### **Competing interests**

The authors declare that they have no competing interests.

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