Salinomycin sensitizes melanoma spheroids containing slow-cycling cells to the effects of arsenic trioxide

（サリノマイシンは細胞周期の遅い細胞を含むメラノーマ・スフェロイドの三酸化ヒ素に対する効果を鋭敏化する）

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Abstract

Recurrence after chemotherapy is a major cause of cancer mortality: subsets of tumor cells evade initial chemotherapy or radiotherapy and survive to re-propagate the tumor. To develop a novel therapeutic approach for melanoma, we applied a non-adhesive culture system which developed spheroids mimicking the properties of melanoma *in vivo*. Subsequently, spheroids involved cells exhibiting clonogenic and slow-cycling properties in addition to chemotherapeutic resistance to doxorubicin. Interestingly, while treatment of spheroids with either salinomycin or As_2O_3 showed limiting effects, a combinatorial treatment was markedly superior to single treatment with each drug. Thus, melanoma spheroids could be a new platform for studying melanoma biology and are likely to provide a clinically relevant target for the novel chemotherapy.

Introduction

Melanoma is the malignancy with the highest increase in incidence in white populations over the past four decades[1]. To date, no single agent has significantly changed survival rates, while the alkylating agent dacarbazine is the only Food and Drug Administration–approved drug for melanoma and has a response rate of 5% to 10%[2] . Moreover, no clinical trials have demonstrated a survival advantage for combination therapy over optimal single-agent therapy. Development of adjuvant therapies that increase survival beyond surgery alone has been therefore urgently needed.

Traditional chemotherapies require fast cycling cells to cause cell death[3]. Slow-cycling cells are therefore less likely to be susceptible to these drugs, suggesting a recurrence mechanism in which slow-cycling cells evade initial chemotherapy and survive to re-propagate tumors. The contribution that slow-cycling populations play in chemotherapy resistance is not well studied, although this characteristic may be a significant factor in tumor recurrence. The better characterization of these therapy-resistant slow-cycling cells is critical for the future development of targeted therapies aimed at achieving more robust and long-lasting responses.

Using the proliferation marker bromodeoxyuridine (BrdU), we have demonstrated that a clonogenic, slow-cycling and doxorubicin-resistant population was enriched in melanoma spheroid cells. Furthermore, we put forward a new combinatorial treatment strategy using arsenic trioxide (As_2O_3) and salinomycin to target chemoresistant cell populations. Our findings pave the way for novel treatment options that will efficiently target chemoresistant cell

populations in melanoma spheroids.

Materials and Methods

Generation of Cells and Spheroids

The mouse melanoma B16-BL6 cell line, supplied by the Cell Resource Center for Biomedical Research, Tohoku University, Japan, was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in the presence of 5% CO_2.

For spheroid formation, single cell suspensions of B16-BL6 cells were plated on 0.5% agarose-coated culture dishes at a density of 3 X 10^5 cells/6 cm dish and maintained in DMEM/Ham's F-12 low osmolality medium in the presence of B27 supplement (Gibco, Grand Island, NY USA), 1000 IU/ml leukemia inhibitory factor (LIF) (Millipore, Billerica, MA USA), 10 ng/ml basic fibroblast growth factor (bFGF) (BD, Franklin Lakes, NJ USA), and 20 ng/ml epidermal growth factor (EGF) (BD, Franklin Lakes, NJ USA), as previously described[4]. On day 3, spheres were dissociated by trypsin-ethylenediaminetetraacetic acid (EDTA) treatment, and maintained in the same medium for another 3 days.

Clonogenic Assay

Quantitation of *in vitro* self-renewal was done by limited dilution assays. Briefly, cells from monolayer culture and spheroid culture were seeded at a ratio of 1 cell per well in 96-well plates to avoid doublets. After 7 days, wells containing colonies stained by 0.5% crystal violet were counted manually under the microscope.

Identification of Slow-cycling Cells

B16-BL6 cells were cultured in DMEM supplemented with 10% FBS with 5μM BrdU(Invitrogen, Carlsbad, CA USA) for 7 days and then maintained in either adherent or spheroids culture medium as described above. On day 6, the trypsin-dissociated monolayer cells and spheroids were centrifuged at 1000rpm and fixed in 4% paraformaldehyde for 4 hours. The pellets were embedded in 5% gelatin, further fixed in 4% paraformaldehyde for 24 hours and embedded in paraffin using standard procedures. For immunohistochemistry, de-paraffinized sections were subjected to heat-induced epitope retrieval using an autoclave pretreatment for 10 min at 121°C in 10mM citrate buffer (pH 6.0). Endogenous peroxidase was blocked with 0.3% hydrogen peroxide for 30 min then incubated with 10% normal goat serum for 30 min at room temperature. The sections were incubated with anti-BrdU rat monoclonal antibody (Abcam, Cambridge, UK) for overnight at 4°C followed with Histofine® Simple Stain™ Mouse MAX-PO (rat) (NICHIREI BIOSCIENCES INC., Tokyo, JPN) for 30 min at room temperature. The sections were visualized with diamino-3,3'benzidine tetrachlorhydrate, counterstained with Mayer's hematoxylin and assessed under light-microscope.

Cell Viability Assay and Apoptosis Assay

Monolayer cells and spheroids were treated with either 0.1 – 1 μg/ml of doxorubicin (Sigma-Aldrich Co., St Louis, MO USA) for 3 hours or 10 μM of As_2O_3 / salinomycin for 24 hrs. After trypsin-dissociation, cells at density of either 5 x 10^3 cells (doxorubicin) or 6 x 10^3 cells (As_2O_3 / salinomycin) were plated to 96-well plates. Cell viability was determined by Alamar-blue® cell viability assay

(Invitrogen, Carlsbad, CA USA) according to the manufacturer's instructions. Fluorescence was measured (excitation/emission: 544/590 nM) on a FLUOROSKAN ASCENT plate reader (Thermo Fisher Scientific Inc., Waltham, MA USA) and the cell viability was calculated by plotting fluorescence emission intensity versus compound concentration.

After the treatment with doxorubicin described above, apoptotic cells were detected by the ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Millipore, Billerica, MA USA) according to the manufacturer's instructions.

Statistics

The experiment was performed in duplicates. Wilcoxon signed-rank test was performed to determine the significance (*p*-values<0.05).

Results

Melanoma spheroid formation and identification of cells capable of self-renewal

To determine whether B16-BL6 melanoma cells could proliferate as non-adherent spheroids, we seeded cells on agarose-coated plastic dishes and cultured in DMEM/Ham's F-12 medium containing B27 supplement and growth factors such as LIF, EGF and bFGF as described above **(Fig. 1a)**. Subsequently, most cells formed small aggregated 24h later **(Fig. 1b)**, and then developed into three-dimensional (3D) balls with a spheroid configuration with a round and smooth contour **(Fig. 1c)** 3days after initial seeding. On day 3, spheres were dissociated by trypsin-EDTA treatment, and maintained in the above-mentioned medium for another 3 days to develop into more tightly packed spheroid **(Fig. 1d)**.

Furthermore, to assess self-renewal capacity of monolayer and spheroid cells, we subjected these cells to cloning to define the ability of a single cell to form a multicellular colony. The limited dilution assay indicated that 29.9 % of spheroid cells were capable of self-renewal and 6.9% of monolayer cells **(Fig. 2)**, suggesting that spheroids contained more populations of clonogenic cells in comparison with monolayer cultures.

Identification of slow cycling cells in spheroids and monolayers

A previous study has suggested that slow-cycling cells can be enriched in spheres when these are cultured in serum-free medium supplemented with adequate mitogens[5)]. We, therefore, aimed to determine

whether slow-cycling cells were enriched in spheroids. After labeling with BrdU, the cells were further cultured in monolayer or spheroid condition, respectively. Over the course of 6 days culture, dividing cells progressively dilute out BrdU and a BrdU-labeled cell population was distinguishable from non-labeled bulk cells by immunohistochemistry. When single cells were analyzed by microscopy, a BrdU-labeled slow-cycling cell population of 9.79 % was detected in spheroid cells and 0.69 % in monolayer cells **(Fig. 3)**, while the differences were not statistically significant, suggesting that spheroid cells contained proportionally more slow-cycling cells in comparison with monolayer cells.

Pattern of chemosensitivity in the different culture conditions

To investigate whether the different culture conditions resulted in a different pattern of chemosensitivity, we examined the effects of doxorubicin on monolayer and spheroid cells. Both cultures were treated with doxorubicin, and cell viability was measured subsequently using an Alamar-blue® cell viability assay. The cell viability assay demonstrated that a chemoresistant cell population of 78, 66.8, and 41.4 % of cells were chemoresistant in spheroid cultures and 52.9, 18.9, and 3.7 % in monolayer cultures at doxorubicin concentrations of 0.1, 0.5 and 1.0 μg/ml, respectively **(Fig. 4)**, suggesting that the spheroid cells are more resistant to doxorubicin in comparison with monolayer cells.

To further analyze the reduced viability of either monolayer or spheroid cells after treatment with doxorubicin, apoptosis of these cell was assessed by TUNEL assay. The results of TUNEL assay demonstrated that an apoptotic cell

population of 8.1% was detected in spheroid cells and 34.5 % in monolayer cells **(Fig. 5)**, suggesting that spheroid cells were more resistant to apoptosis induction in comparison with monolayer cells.

Targeted treatment of distinct populations of spheroid cells

Spheroids are enriched for slow-cycling cells and are resistant to classical chemotherapeutic drugs doxorubicin as described above. Therefore, to develop a novel therapeutic approach, we treated spheroid cells with either As_2O_3 or salinomycin individually as well as in combination. The cell viability assay demonstrated that a chemoresistant cell population of 0.4 % was present after combination treatment whereas 131.5 % (As_2O_3) and 33 % (salinomycin) of cells where present after single treatment **(Fig. 6)**, suggesting that a combinatorial treatment with As_2O_3 and salinomycin was superior to single treatment with each drug.

Discussion

Although monolayer cultures of human cell lines is probably the most extensively used model system for detection of new molecules that might further develop into cancer drugs, this model does not reflect the pathophysiology of solid tumors. In contrast, cells grown as spheroids more closely mimic solid tumors and are thus a way to get closer to the clinical situation when studying cancer drugs[6]**.** In the present investigation, melanoma cells were grown in a non-adherent culture system, with the aim to more closely mimic solid tumors *in vivo* with respect to chemoresistance. Our data demonstrated that spheroids involved cells with more clonogenic, slow-cycling and chemoresistant characteristics in comparison with monolayer cells and suggested that these cells in solid cancer may differ in therapy response to the bulk cells. Our model that could reflect the clinical activity of a drug would be of substantial value in the development of novel cancer drugs.

The phenomenon of slow-cycling characteristics has been observed in normal adult stem cells in many different tissues such as the skin, the intestine and the hematopoietic system[7]. These slow-cycling cells have been proposed to be important for life-long self-renewal and for the generation of the different cellular lineages[8]. Moreover, evidence for such chemoresistant abilities is observed in normal skin tissue where slow-cycling cells in the bulge of hair follicles survive chemotherapy to regenerate the follicle[9]. It remains unclear what mechanisms are involved in the biological properties of spheroids, although several studies have suggested roles for certain signaling pathways play involved in cell growth, metastatic potential and chemoresistance[10,11,12].

It is unclear whether the slow cycling cells enriched in melanoma spheroid culture are to the same as the so-called cancer stem cells (CSCs) or tumor-initiating cells (TICs) reported previously[13]. The theory of CSCs/TICs states that a small subset of cancer cells has the exclusive capacity to divide equally into both the CSCs/TICs pool and more differentiated cell lineages[14]. Given the clonogenic and chemoresistant properties, there might be a partial correlation between slow-cycling cells and CSCs/TICs. However, it is beyond the scope of the present article to further discuss the CSCs/TICs hypothesis with respect to the ongoing controversy related to the identification of sufficient markers to define the CSCs/TICs lineage. The approaches described here may provide a basis for identification of cells with CSCs/TICs characteristics within 3D tumor spheroids, allowing these relatively rare populations of cells to be analyzed.

Arsenic is a potent carcinogen, and arsenic exposure is well documented to lead to the development of various types of solid tumors. Notably, there are also studies that have shown that one form of arsenic, As_2O_3, exhibits potent anti-tumor activities for acute promyelocytic leukemia as well as other hematologic malignancies such as myelodysplastic syndrome and multiple myeloma[15]. As_2O_3 acts on cells through a variety of mechanisms, influencing numerous signal transduction pathways. An important cellular event that occurs during apoptosis induction with As_2O_3 involves elevation of reactive oxygen species (ROS), which leads to decreases in the mitochondrial membrane potential, resulting cytochrome c release and activation of the caspase cascade[16]. This appears to be a common mechanism of induction of cell death

in diverse cellular backgrounds. Beyond regulation of common cellular pathways in different types of tumors cells, the present results are consistent with the previous work demonstrating that As_2O_3 inhibits proliferation of melanoma cells *in vitro*[17] . However, As_2O_3 was not effective in the treatment of acute promyelocytic leukemia at the plasma concentration tested (5.54 μM to 7.30 μM), although the potential exists for synergism with other agents to provide enhanced therapeutic benefits.

Salinomycin, which has been used as an agricultural antibiotic to prevent coccidiosis, was recently shown to significantly reduce cell viability of human cancer cells with stem cell-like properties resistant to common chemotherapeutic drug[18]. Salinomycin functions as a transmembrane potassium ionophore that is able to overcome ATP-binding cassette (ABC) transporter mediated multidrug resistance[19]. Thus, these characteristics of salinomycin have the potential to be exploited to increasingly sensitize cells to anticancer drugs as part of combination chemotherapy. Hence, we investigated the synergic effect of salinomycin on the reduced viability of melanoma cells induced by As_2O_3 treatment. The present results demonstrated enhanced cell death, if administered in combination with As_2O_3. Such combinations in clinical appplication may result in enough antitumor activity with acceptable tolerability.

The design of rational therapeutics targeting key players in disease pathways will certainly be the focus of translational research in the coming years. Our ongoing experimental trials using a spheroid model provide hope for development of new therapeutic approaches for melanoma. Their rational

development will require considerable additional efforts to understand the many molecular actions in the cellular events.

References

1. Garbe C, Leiter U. Melanoma epidemiology and trends. Clin Dermatol 27: 3-9, 2009

2. Serrone L, Zeuli M, Sega FM, Cognetti F. Dacarbazine-based chemotherapy for metastatic melanoma: thirty-year experience overview. J Exp Clin Cancer Res 19: 21–34, 2000

3. Moore N, Houghton J, Lyle S. Slow-cycling therapy-resistant cancer cells. Stem Cells Dev 21: 1822-1830, 2012

4. Rappa G, Mercapide J, Anzanello F, et al. Growth of cancer cell lines under stem cell-like conditions has the potential to unveil therapeutic targets. Exp Cell Res 314: 2110–2122, 2008

5. Wang Y, Sacchetti A, van Dijk MR, van der Zee M, van der Horst PH, Joosten R, Burger CW, Grootegoed JA, Blok LJ, Fodde R. Identification of quiescent, stem-like cells in the distal female reproductive tract. PLoS One 7: e40691, 2012

6. Hirschhaeuser F, Menne H, Dittfeld C, West J, Mueller-Klieser W, Kunz-Schughart LA. Multicellular tumor spheroids: an underestimated tool is catching up again. J Biotechnol 148: 3-15, 2010

7. Fuchs E. The tortoise and the hair: slow-cycling cells in the stem cell race. Cell. 137: 811-819, 2009

8. Fuchs E, Chen T. A matter of life and death: self-renewal in stem cells. EMBO Rep. 14: 39-48, 2013

9. Cotsarelis G, Millar SE. Towards a molecular understanding of hair loss and its treatment. Trends in Molecular Medicine 7: 293-301, 2001

10. Ray A, Meng E, Reed E, Shevde LA, Rocconi RP. Hedgehog signaling pathway regulates the growth of ovarian cancer spheroid forming cells. Int J Oncol 39: 797-804, 2011

11. Peart TM, Correa RJ, Valdes YR, Dimattia GE, Shepherd TG. BMP signalling controls the malignant potential of ascites-derived human epithelial ovarian cancer spheroids via AKT kinase activation. Clin Exp Metastasis 29: 293-313, 2012

12. Kim H, Phung Y, Ho M. Changes in global gene expression associated with 3D structure of tumors: an ex vivo matrix-free mesothelioma spheroid model. PLoS One 7: e39556, 2012

13. Roesch A, Fukunaga-Kalabis M, Schmidt EC, Zabierowski SE, Brafford PA, Vultur A, Basu D, Gimotty P, Vogt T, Herlyn M. A temporarily distinct subpopulation of slow-cycling melanoma cells is required for continuous tumor growth. Cell 141: 583-594, 2010

14. Francipane MG, Chandler J, Lagasse E. Cancer Stem Cells: A Moving Target. Curr Pathobiol Rep 1: 111-118, 2013

15. Tomita A, Kiyoi H, Naoe T. Mechanisms of action and resistance to all-trans retinoic acid (ATRA) and arsenic trioxide (As_2O_3) in acute promyelocytic leukemia. Int J Hematol 97: 717-725, 2013

16. Platanias LC. Biological responses to arsenic compounds. J Biol Chem. 284:18583-185837, 2009

17. Hiwatashi Y, Tadokoro H, Henmi K, Arai M, Kaise T, Tanaka S, Hirano T. Antiproliferative and anti-invasive effects of inorganic and organic arsenic compounds on human and murine melanoma cells in vitro. J Pharm

Pharmacol.63:1202-1210, 2011

18. Gupta PB, Onder TT, Jiang G, Tao K, Kuperwasser C, Weinberg RA, Lander ES. Identification of selective inhibitors of cancer stem cells by high-throughput screening. Cell.138: 645-659. 2009

19. Fuchs D, Daniel V, Sadeghi M, Opelz G, Naujokat C. Salinomycin overcomes ABC transporter-mediated multidrug and apoptosis resistance in human leukemia stem cell-like KG-1a cells. Biochem Biophys Res Commun 394:1098-1104, 2010

Figure Legends

Fig. 1: Morphology of spheroid cells. Mouse melanoma B16-BL6 cells were seeded onto a non-adhesive culture dish (a). The cells formed small aggregates after 24h (b) and large balls with a spheroid configuration after 72h (c). After trypsin treatment, cells were maintained for another 3 days to develop into more tightly packed structures (d).

Fig. 2: Spheroids contained proportionally more clonogenic cells than monolayers. Dissociated cells were seeded at single cell 96-well plates, cultured for 7 days, and stained with crystal violet to visualize colony growth.

Fig. 3: Spheroids contained proportionally more slow-cycling cells than monolayer cultures. After labeling with BrdU, cells were maintained in either a non-adhesive (a) or an adhesive culture system (b). The number of slow-cycling cell population in spheroids was compared with that in monolayers (c).

Fig. 4: Spheroids were more resistant to doxorubicin in comparison with monolayers. After treatment with different concentrations of doxorubicin, cell viability was measured by Alamar-blue® cell viability assay.

Fig. 5: Spheroids were more resistant to apoptosis induction in comparison with monolayers. After treatment with doxorubicin, apoptotic cells in either spheroids (a) or monolayers (b) were measured by TUNEL assay (c).

Fig. 6: A combinatorial treatment with As_2O_3 and salinomycin was superior to single treatment with each drug. After treatment with As_2O_3 and salinomycin as well as in combination, cell viability was measured by Alamar-blue® cell viability assay.

Fig.1

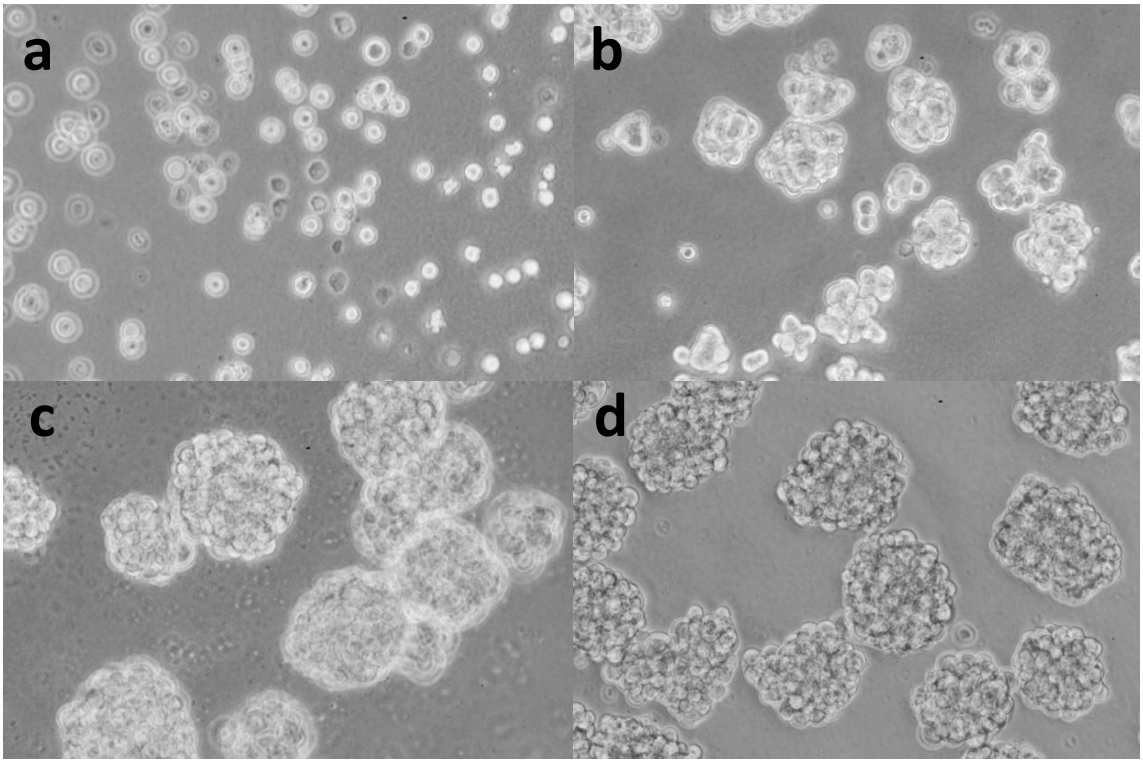


Fig.2

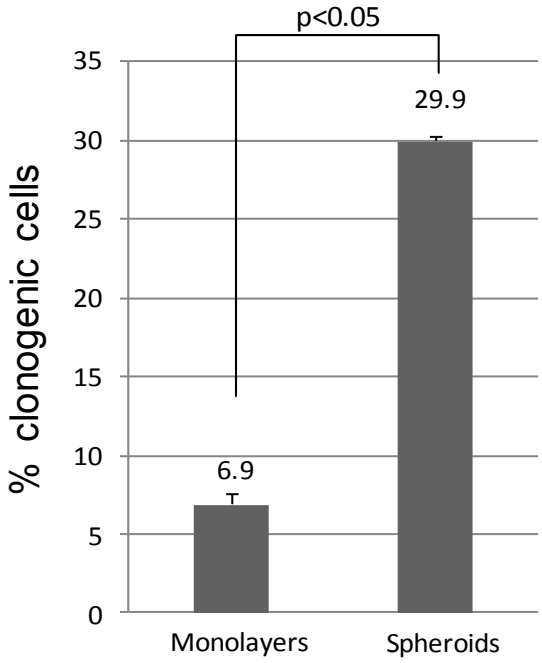
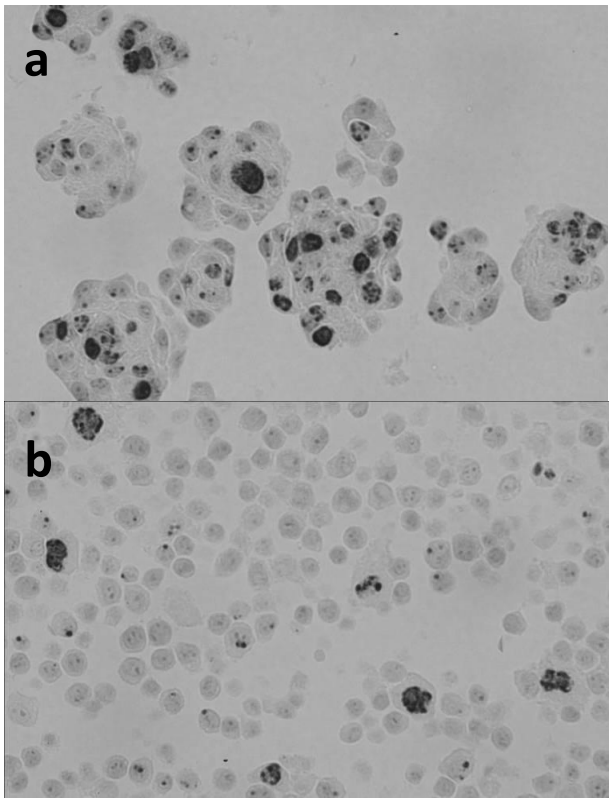
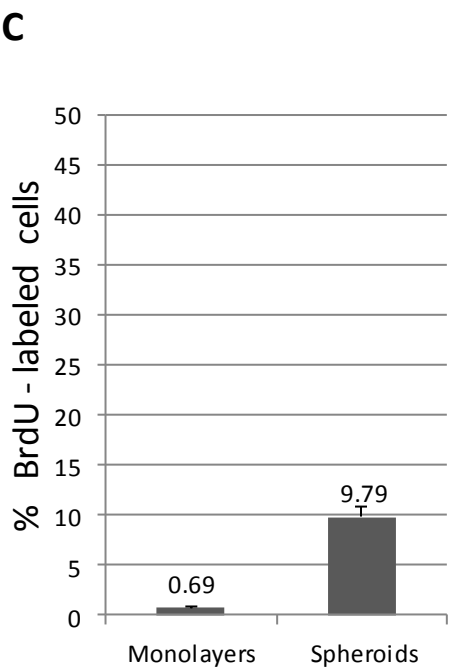

p<0.05
29.9
6.9
% clonogenic cells
35
30
25
20
15
10
5
0
Monolayers
Spheroids

Fig.3


a
b


c
% BrdU - labeled cells
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45
40
35
30
25
20
15
10
5
0
9.79
0.69
Monolayers
Spheroids

Fig.4

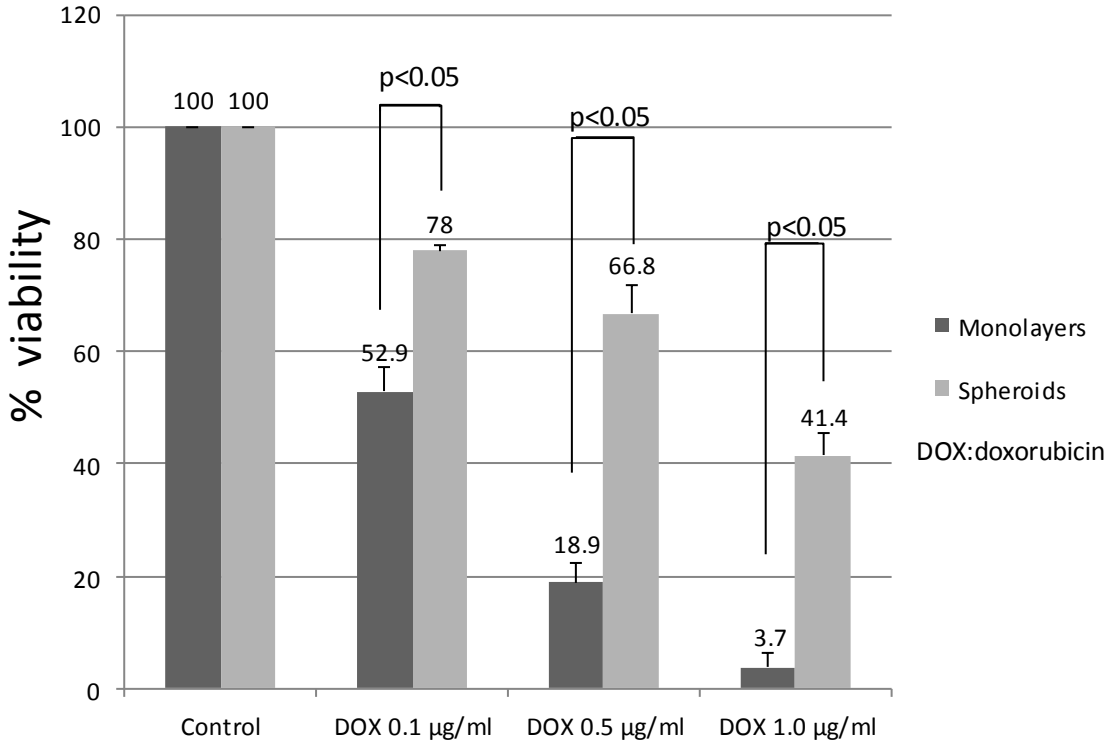

120
100
80
60
40
20
0
% viability
100 100
p<0.05
p<0.05
p<0.05
78
66.8
52.9
18.9
41.4
3.7
Control
DOX 0.1 μg/ml
DOX 0.5 μg/ml
DOX 1.0 μg/ml
Monolayers
Spheroids
DOX:doxorubicin

Fig.5

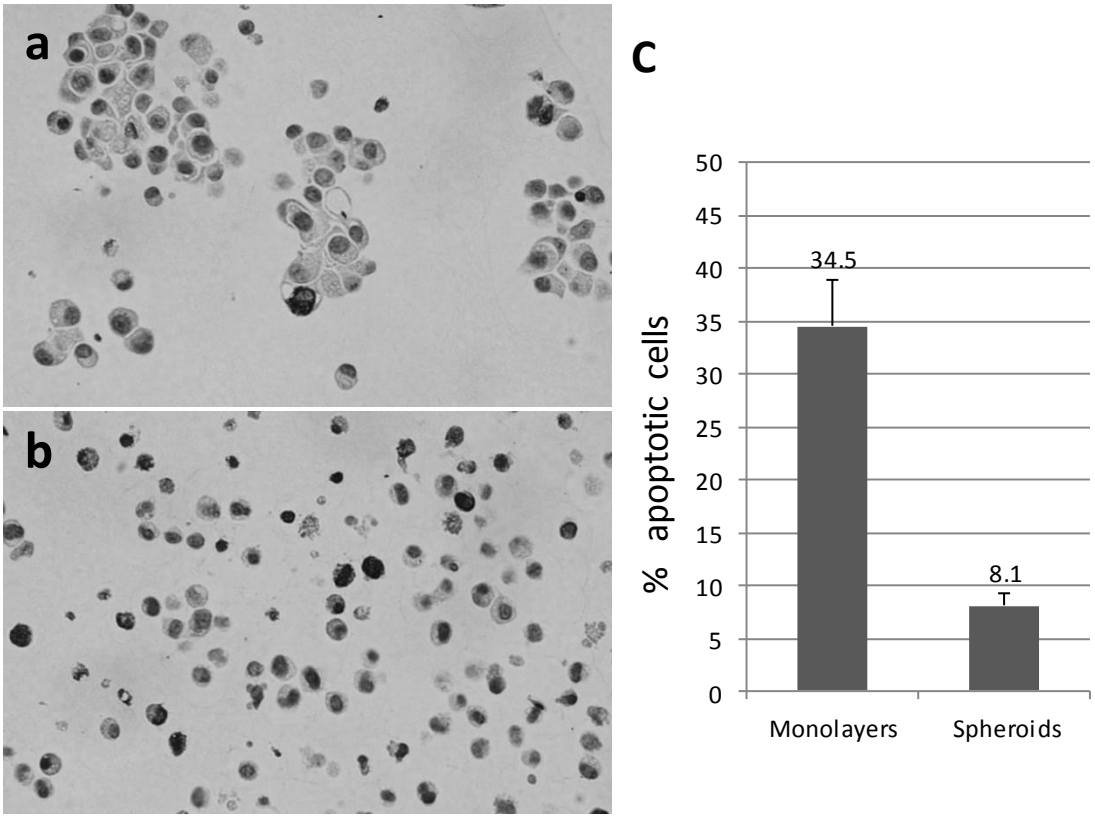
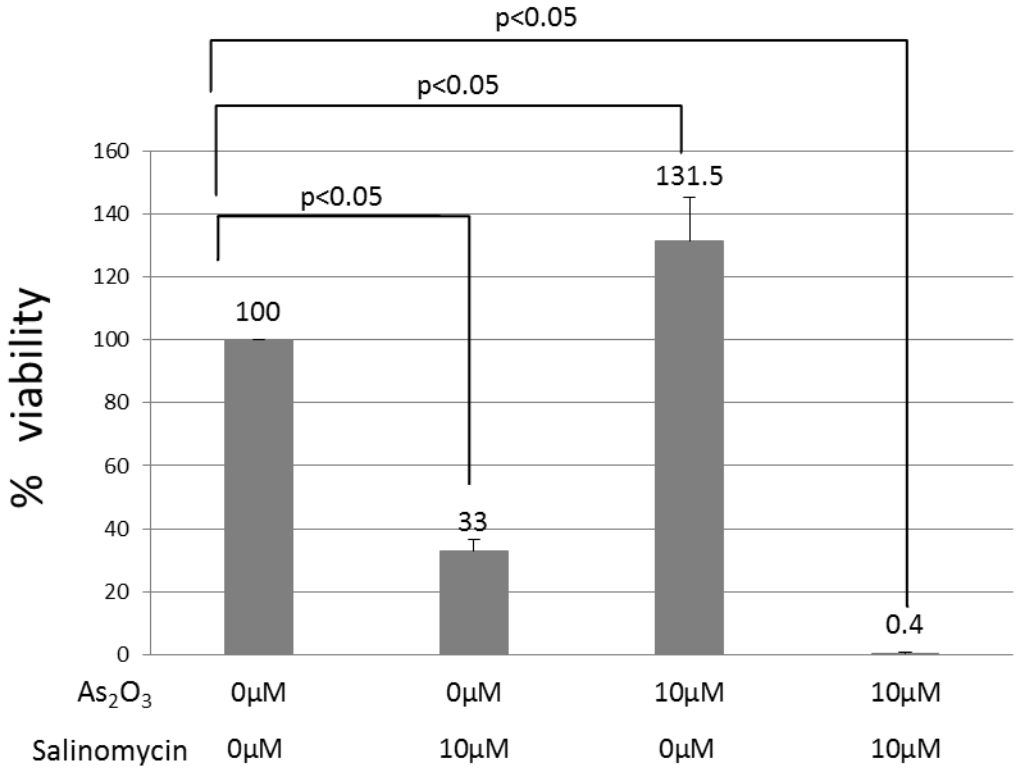

a
b
c
% apoptotic cells
50
45
40
35
30
25
20
15
10
5
0
34.5
8.1
Monolayers
Spheroids

Fig.6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p<0.05
p<0.05
160
140
120
100
80
60
40
20
0
% viability
100
33
131.5
0.4
As2O3
0μM
0μM
10μM
10μM
Salinomycin
0μM
10μM
0μM
10μM