Tumour Stem Cell Enrichment by Anticancer Drugs: A Potential Mechanism of Tumour Recurrence

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Abstract

Tumour recurrence after chemotherapy is a serious clinical problem. An emerging concept in tumour biology is the cancer stem cell hypothesis, which emphasises the importance of rare tumour stem cell-like cells to reinitiate the tumour even after a successful elimination of the primary tumour mass by surgery, chemotherapy or radiotherapy. We employed live cell tools to monitor caspase-mediated cell death or survival after in vitro drug treatment to investigate events associated with enrichment of CSCs in breast and colon cancer cells. We provide evidence for rare escape of cells from drug-induced caspase activation that enriches cells with stem cell-like cells. Interestingly, an intermediate senescent-dominating population was evident during the transition and the post-senescent; drug-surviving cells were enriched with dye efflux cells with embryonic stem cell markers. Since senescence-escaped stable colonies generated are enriched with stem cell-like phenotype from natural tumour cell models and also stably express sensitive caspase sensor, in the future they can be utilised for screening compounds that target them.

Keywords

Tumour stem cells • Anticancer drugs • Drug resistance • Side population cells • Senescence • Apoptosis

2.1 Introduction

Most chemotherapeutics or target-specific drugs used for the treatment of cancer are capable of reducing tumour burden with immediate promising tumour-free survival. However, this initial successful treatment outcome is most often followed by tumour recurrence in a subset of tumours. The accumulating evidence attributes quiescent tumour stem cell-like cells within the heterogeneous tumour population as the cellular origin for tumour recurrence (Gangemi et al. 2009; Milas and Hittelman 2009). This theory is further strengthened by the recent reports suggesting that tumour stem cell-like cells are inherently resistant to conventional antitumour agents (Singh and

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Settleman 2010; Ribatti 2012). The concept of tumour stem cells, even though known for many years, experimentally proved for its existence first time in leukaemia (Lapidot et al. 1994). Subsequently both surface marker and functional trait-based identification of this rare population were documented in a variety of solid tumours including breast, colon and CNS (Korkaya et al. 2011; Lathia et al. 2011). Cancer stem cells, just like normal stem cells, exist as quiescent phenotype that renders them resistant to conventional cytotoxic agents that often exerts more damage to proliferating cells in the bulk tumour. However, several recent studies indicate that cancer stem cell phenotype can be induced by a variety of stimuli. Epithelial–mesenchymal transition by TGF-β treatment or by ectopically expressed transcription factors like Twist and Snail and overexpression of pluripotency-inducing factors also contributes for the induction of stem cell phenotype (Ksiazkiewicz et al. 2012; Zhu et al. 2012; Krantz et al. 2012; Kong et al. 2011; Floor et al. 2011). In addition, several reports attribute a role for certain drugs to induce EMT as well as tumour stem cell phenotype as a potential mechanism for drug failure. We have recently showed evidence for rare escape of tumour cells from drug-induced cell death, after an intermediate stay in a noncycling senescent stage followed by unstable multiplication often characterised with spontaneous cell death (Achuthan et al. 2011). The cells which escaped from drug-induced toxicity showed increased resistance to cell death by caspase activation and tumour stem cell-like phenotype such as CD133 and Oct4 expression. Extensive functional characterisation of the drug-escaped cells revealed that their drug resistance phenotype is closely related to low levels of intracellular reactive oxygen species (ROS) subsequent to reactivation of antioxidant enzymes (Achuthan et al. 2011). The drug-induced tumour stem cell enrichment, even though not well defined, carries tremendous implications in the understanding of drug failure, tumour recurrence and metastasis and also provides opportunities for drug intervention. Here, we further provide evidence for the existence of rare tumour stem cell-like cells in multiple tumour cells and their enrichment after drug exposure. The colon cancer cells used in the current study also showed evidence for intermediate senescent stage and indication for rare entry into cell cycle from drug-induced senescence. Currently, it is not clear whether the enrichment is due to the escape of rare cells from drug treatment or it is induced by the drugs either by genetic or epigenetic signalling. However, the drug-escaped population remains as a potential cell source to identify agents that target physiologically relevant drug-resistant cells with stem cell-like property. Since tumour stem cell-like cells are very rare and difficult to identify from natural cancer cell models, it remains as a challenge to develop cell-based assays to identify compounds that target them (Gupta et al. 2009). Since the methods described here enrich the cells with stem cell-like phenotype from natural tumour cell models, that can be utilised for screening compounds that targeting them.

2.2 Materials and Methods

2.2.1 Cell Lines

Human breast tumour cell lines MCF-7; colon cancer cell lines SW480, SW620 and HCT11; and cervical cancer cell lines SiHa, HeLa and C3AA were obtained from ATCC, USA. All cancer cell lines were maintained in RPMI medium supplemented with 1 % penicillin–streptomycin and 10 % foetal bovine serum. All cell lines were incubated in a humidified incubator at 37 ºC supplied with 5 % carbon dioxide.

2.2.2 Side Population (SP) Analysis

The cell suspensions were labelled with Hoechst 33342 dye (Invitrogen, USA) using methods as previously described (Sobhan et al. 2012; Bleau et al. 2009). Briefly, cells were resuspended at 1×10^6/mL in prewarmed RPMI with 2 % FBS and 10 mM HEPES buffer containing 5 μg/mL of Hoechst 33342 in the presence or absence of verapamil at 37 °C for 90 min. Then, the cells were washed and resuspended in ice-cold HBSS

containing 2 % FBS and 10 mM HEPES. Propidium iodide at a final concentration of 2 μg/mL was added to the cells to gate viable cells. Side population analyses were done on FACSAria I (BD, USA).

2.2.3 Drug Treatment

Cells were seeded at a density of 2×10^5 cells/well in 12-well plates containing DMEM supplemented with 10 % FBS. After overnight incubation, different drugs were added to each cell line and maintained at 37 ºC in a water-saturated atmosphere containing 5 % CO_2. Medium replacement was done every fourth day with fresh drug-containing medium. The duration of exposure to each drug was about 3–6 weeks.

2.2.4 Live Cell Visualisation of Cell Death and Survival Using Cells Expressing Caspase-3 Fluorescence Resonance Energy Transfer (FRET) Probe

We have previously employed caspase sensor expression vector, SCAT 3.1, which consists of ECFP and EYFP (Venus), separated by caspase cleavage site – DEVD – to generate stable clones in breast cancer cell lines (Joseph et al. 2011). This approach enabled us to visualise immediate cell death by caspase activation upon drug treatment and also tracking of the surviving cells. Since the surviving clones were enriched with cells with tumour stem cell-like properties including surface phenotype of CD133 and CD44 as well as functional traits low ROS, Oct4 induction and reactivation of antioxidant machinery, we hypothesised that if proved in more cells, the emerging cell population may form a better tool for identifying agents that target them. Keeping this in mind, a new-generation FRET probe for caspase activation was employed to generate stable clones. This vector consists of Ametrine as the donor fluorescent protein and Tomato as the acceptor protein linked in-between the sequence, DEVD, with a nuclear exclusion signal (NES).

Stable clones were generated with this vector both in MCF-7 and colon cancer cell line SW480 by transfections followed by flow sorting to get cells that are enriched with high FRET efficiency using FACSAria I (BD, USA). For FRET imaging by microscopy, cells were seeded on a chambered cover glass (Nunc International, NY) and maintained in live cell incubation chamber (Tokai-Hit, Japan) at 37 ºC with 5 % CO_2 for indicated time periods. Images were collected using an Epi fluorescent microscope TE-2000E (Nikon, Japan) at regular intervals. Single excitations of pmAmetrine at 387/11 and dual emission at 535/30 and 585/29 were collected using automated excitation and emission filter wheel controlled by NIS software (Nikon, Tokyo) as described (Ai et al. 2008).

2.2.5 Assessment of Cell Cycle Progression in Live Cells

For live cell visualisation of cell cycle progression, G1-specific fluorescent component of FUCCI, Cdt1–Kusabira Orange fusion construct and G2-specific green fluorescent marker Geminin–Azami Green were employed to generate stable clones (Sakaue-Sawano et al. 2008). Cells were transfected with the expression vector Cdt1–KO and Geminin–Azami Green followed by selection in G418. The cell expressing both red and green fluorescent proteins was isolated by flow sorting. Stable integration and cell cycle-specific fluorescence were evaluated by live cell imaging. The cells were seeded on chambered cover glass for live cell imaging as described above.

2.3 Results

2.3.1 Side Population Cells in Established Cancer Cell Lines

Despite increasing number of reports substantiating the existence of tumour stem cell-like cells in multiple tumour types, their identification

remains as a challenge due to lack of a common surface marker. In the last decade, contributions from several laboratories revealed importance of several phenotypes to identify them from multiple tumour cell models. $CD44^{+}/CD24^{low}$ and Aldefluor-high and CD133-high fractions were reported as enriched with cells with tumour-initiating potential in breast, colon, neural and prostate cancers (Scheel and Weinberg 2011; Fang et al. 2012; Singh and Dirks 2007). Functional features like low reactive oxygen species and drug efflux were also explored as valuable marker of tumour stem cell-like cells (Wang et al. 2010). Another widely used method, side population analysis utilises the ability of cells to exclude the fluorescent dye Hoechst, owing to the preferred expression of p-glycoprotein in stem cell-like compartment (Bleau et al. 2009; Sobhan et al. 2012; Engelmann et al. 2008). In an effort to characterise side population cells, we have employed multiple tumour cells of different tissue origin to determine the steady-state level of side population cells by flow cytometry. A typical scatter plot of side population analysis in the colon cancer cell line SW620 is shown in Fig. 2.1a. The percentage of cells gated as side population in the scatter plot is diminished in verapamil- treated cell population substantiating their dependence on drug efflux transporters. The histogram shown in Fig. 2.1b is the quantitative representation of the average percentage of side population cells in multiple tumour cells as determined by FACS ($n = 3$).

The results shown above indicate that cancer cells maintained continuously in culture will retain a small fraction of cells with drug efflux property that can be identified by FACS. As shown in the histogram, most cells retain a small fraction of SP irrespective of the tissue origin and that varies significantly between cells. Several reports as well as our previous studies substantiated the drug-resistant nature of side population cells compared to the main population (Sobhan et al. 2012; Achuthan et al. 2011; Gangemi et al. 2009).

2.3.2 Side Population Cells Are Enriched in Drug-Evaded Cells

In a previous study, we have shown that exposure of breast cancer cells to multiple antitumour agents generates drug-resistant clonal expansion in a delayed manner (Achuthan et al. 2011). Currently, it is not clear whether this phenomenon is unique to breast cancer cells or general in nature. So we have employed colon cancer cells SW480, SW620 and HCT 116 and exposed them to camptothecin and doxorubicin for two cycles with an interval of 3 days. Then the plates were maintained in drug-free medium to allow expansion of colonies as described previously. At the end of 30–45 days, the emerging colonies were trypsinised and analysed side population by FACS. Both the drugs significantly increased the percentage of cells with drug efflux property in all the cells. The results from SW480 are shown on the graph in Fig. 2.2a. A representative scatter plot of SW480 cells generated after treatment with doxorubicin is also shown (Fig. 2.2b). The results shown above prove that side population cells are enriched after multiple cycles of drug exposure in colon cancer cells. This is consistent with similar observation noticed in breast cancer cells (Achuthan et al. 2011). Overall, these results indicate for a possible unique general signalling in cancer cells that enriches cells with considerable phenotype transition, rendering them resistant to drugs during exposure to cytotoxic agents.

2.3.3 A FRET-Based Caspase Sensor to Track the Emergence of Drug-Resistant Colonies

Since most antitumour agents kill cancer cells by apoptosis involving activation of caspases, we have previously employed stable breast cancer cell lines expressing caspase-specific FRET probe ECFP–DEVD–EYFP to track caspase-evading cells in breast cancer cell line (Joseph

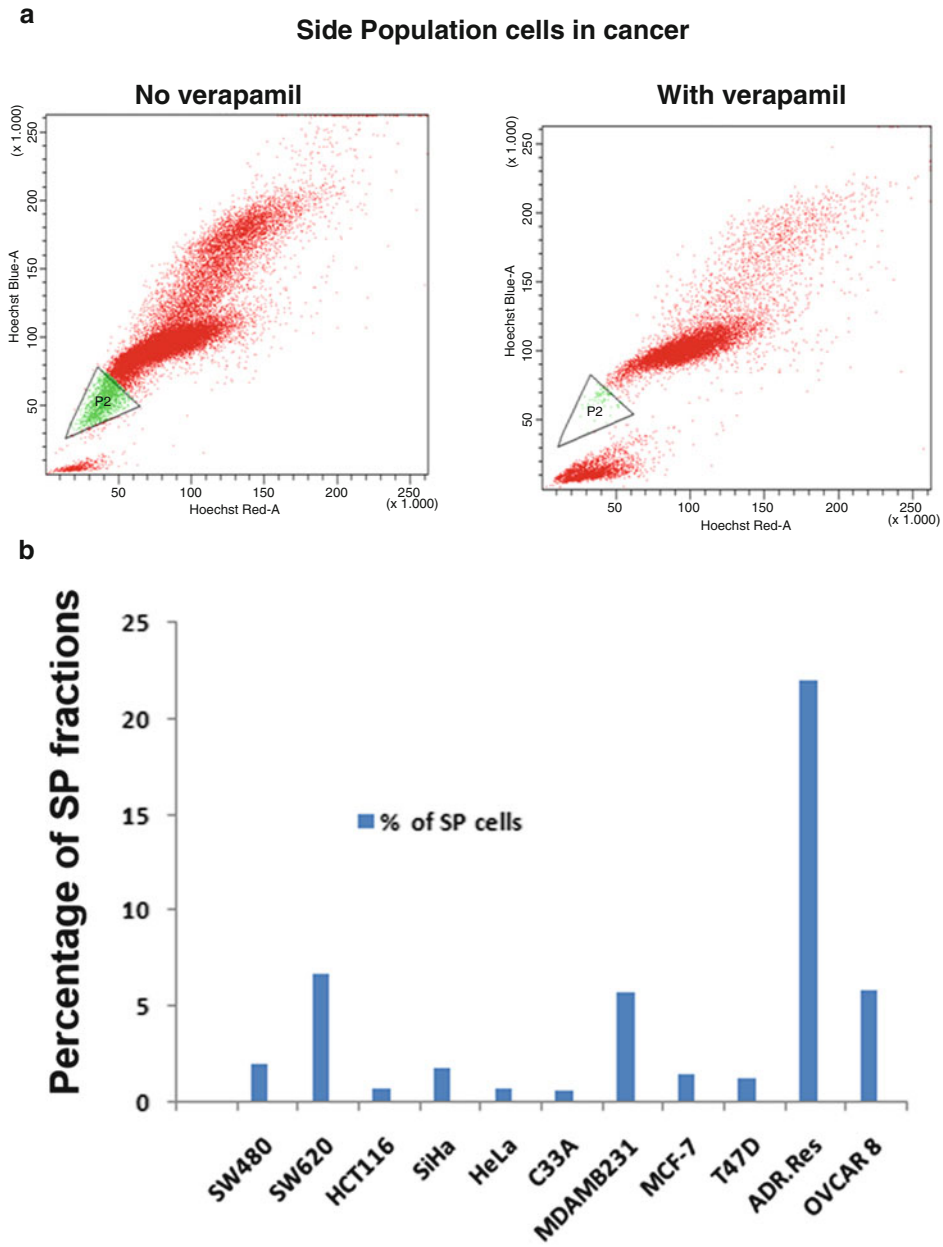

Fig. 2.1 *Side population cells were present in established cancer cell lines*. (**a**) SW620 cells were stained with Hoechst 33342 dye in the presence (*right*) or absence (*left*) of 50 μmol/L verapamil and analysed by flow cytometry. (**b**) Indicated cell lines were stained with Hoechst for side population analysis as described in materials and methods. Average percentage of SP cells gated in multiple cancer cells is shown as *bar diagram*

et al. 2011). This model was quite useful to visualise the emerging colonies and also to study their drug response. The results shown above from the colon cancer cell lines indicate that drug-escaped cells are enriched with high fraction of side population cells, a population with stem cell-like properties. So, we hypothesised that since the drug treatment enriches cells with stem

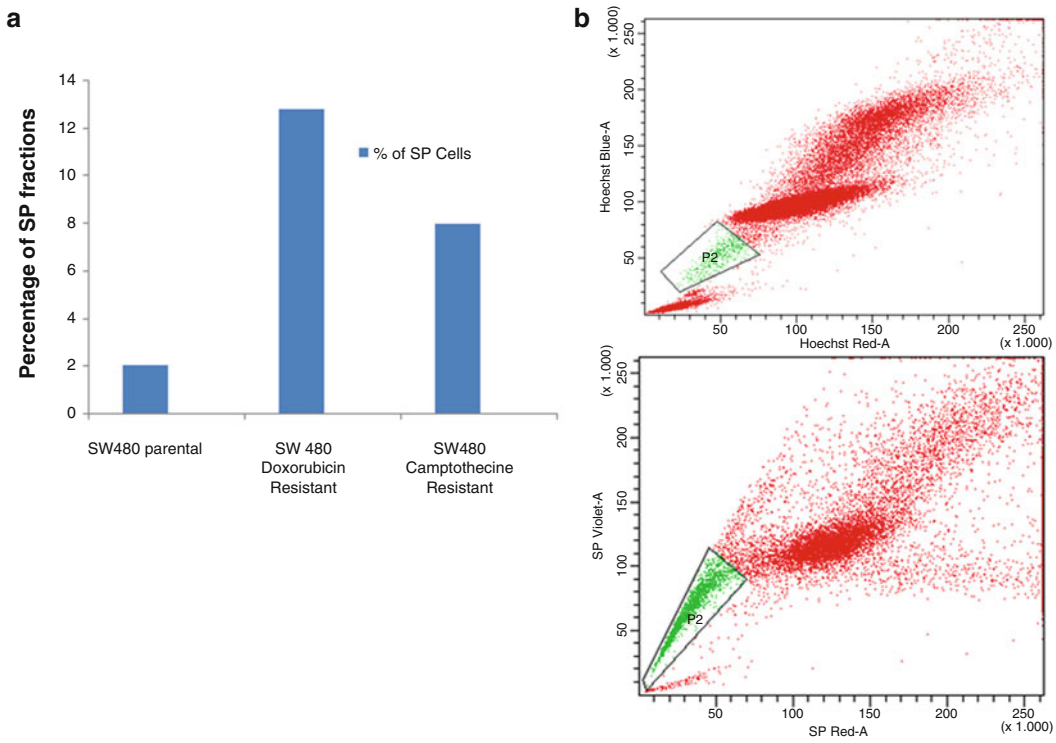

Fig. 2.2 *Drug-induced enrichment of side population cells*. (**a**) SW480 cells were exposed to doxorubicin and camptothecin and drug-escaped cells were generated as described. The side population analysis was done as described. (**b**) A representative scatter plot of SW480 cells generated after treatment with doxorubicin and the parental SW480 is shown

cell-like properties, this enriched fraction may form a better cell source for screening drugs that specifically target them. The FRET-based caspase sensor cell system is highly adaptable for high-throughput image-based drug screening (Joseph et al. 2011). So we have employed a sensitive system of FRET-based caspase activation tool Ametrine–DEVD–Tomato (Ai et al. 2008). Initially, we developed breast cancer cell line, MCF-7, expressing Ametrine–DEVD–Tomato stable cell line as described. The colon cancer SW480 was also employed to study the generation of drug-tolerant cells.

Consistent with our earlier results in breast cancer cells, it was seen that after an initial phase of massive cell death by caspase activation, few cells enter into noncycling quiescent-like state with large, flattened morphology, a characteristic feature of senescent cells in SW480 cells also. A representative live cell image of senescent cells generated after doxorubicin treatment in MCF-7 and SW480 cells expressing Ametrine–DEVD–Tomato is given in Fig. 2.3a, b. As evident from the figure, few cells with flattened senescent morphology are flanked by small dividing cells indicating occasional cell cycle entry during the transition.

2.3.4 Senescence and Delayed Cell Cycle Entry in Drug-Treated Cells

Even though late outgrowth colonies were known to emerge from the drug-treated population after an intermediate senescent-dominating cell population, currently it is not clear whether the cells were generated from senescent cells. Cellular senescence is a general response of normal cells undergoing limited replication before

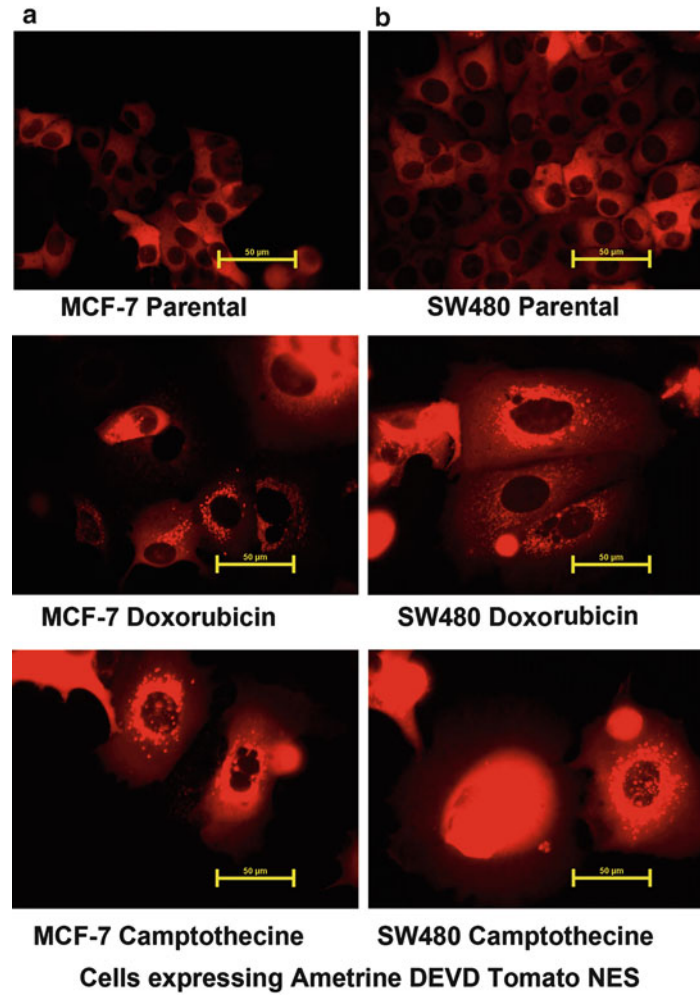

Fig. 2.3 *Drug-induced senescence and cell cycle entry*. (**a**) A representative live cell image of senescent cells generated after doxorubicin treatment in MCF-7 expressing Ametrine–DEVD–Tomato. (**b**) A representative live cell image of senescent cells generated after doxorubicin treatment in SW480 cells expressing Ametrine–DEVD–Tomato NES

entering into a terminally growth-arrested state attributed to telomere attrition. Tumour cells, though considered as immortal, also retain the capacity to undergo senescence in response to genotoxic stress, radiotherapy and chemotherapy. Senescent cells are generally resistant to apoptosis and can serve as reservoirs of secreted factors for mitogenic and angiogenic activity; however, the evidence for their entry into cell cycle is not well established. In order to prove cell cycle entry, we have employed the cell cycle indicator in live cell, FUCCI, as described (Sakaue-Sawano et al. 2008). The SW480 cells stably expressing cytochrome c-EGFP were stably transfected with cdt–KO and Geminin–Azami Green. Cells in the G1 phase show red cdt fluorescence in the nucleus and the G2 phase with Geminin–Green fluorescence as described (Sakaue-Sawano et al. 2008). The S-phase cells expressed both cdt and Geminin in the nucleus. This approach helped to identify the S-phase fractions during the course of drug treatment. Close observation of multiple wells revealed rare S-phase entry by few cells with senescent morphology. A representative image is shown in Fig. 2.4. As seen from the figure around senescent cells, few cells show expression of both cdt and Azami Green substantiating S-phase entry.

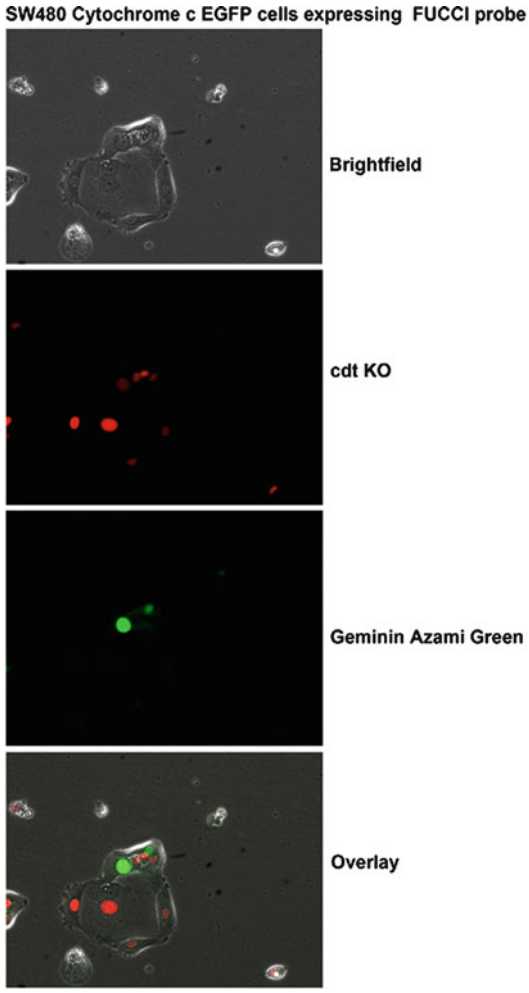

Fig. 2.4 *Evidence for cell cycle entry from senescence*. The SW480 cells stably expressing cytochrome c-EGFP were stably transfected with cdt–KO and Geminin–Azami Green. The cells were exposed to three cycles of doxorubicin and allowed to recover in drug-free medium. As seen from the figure around senescent cells, few cells show both cdt and Azami Green indicating they are in S phase

2.3.5 Drug-Surviving Cells Are Resistant to Drugs and Enriched with Tumour Stem Cell-Like Properties: Potential Applications in Drug Screening

Results from the above studies indicate possible generation of drug-resistant cells from senescent cells by occasional reentry into cell cycle. Moreover, the drug-escaped populations were highly enriched in side population cells as well as cells with stem cell properties. To examine whether drug-surviving cells displayed resistance to drugs, the cells expressing the FRET probes were exposed to drugs, and drug-tolerant cells were generated as described. Both parental and drug-tolerant cells were exposed to different anticancer agents, and caspase activation was analysed by ratio imaging as described in the materials and methods. A representative ratio imaging of doxorubicin-resistant and parental cells to three different drugs is shown in Fig. 2.5a–c. Compared to parental cells, drug-escaped cell showed marked resistance to caspase activation. Most cells failed to change the ratio of Ametrine–Tomato compared to the parental cells.

Our results again indicate a role for drug-induced senescence in tumour recurrence through clonal expansion of multidrug-resistant cells both in breast and colon cancer cells. The ratio imaging described here using the Ametrine–DEVD–Tomato NES-stable cells is highly adaptable for high-throughput imaging, and the drug-escaped cell model has potential applications in drug screening, specially to identify compounds that target cells with tumour stem cell-like properties.

2.4 Discussion

Treatment failure and tumour recurrence after chemo- or radiotherapy remain as the major challenge in successful cancer treatment. It has been increasingly realised that the conventional treatment modalities are not enough to ensure complete eradication of tumour cells even with targeted therapies. In general, cancer that relapses after treatment remains resistant to most chemotherapeutic agents even to the different chemical compounds than used for initial treatment. Multiple hypotheses were put forwarded to explain tumour recurrence and their resistance. Cancer has been viewed as a highly heterogeneous population, and this heterogeneity also brings differential response to drugs. This heterogeneity of response may allow some cells to escape drug-induced cytotoxic stimuli to ensure

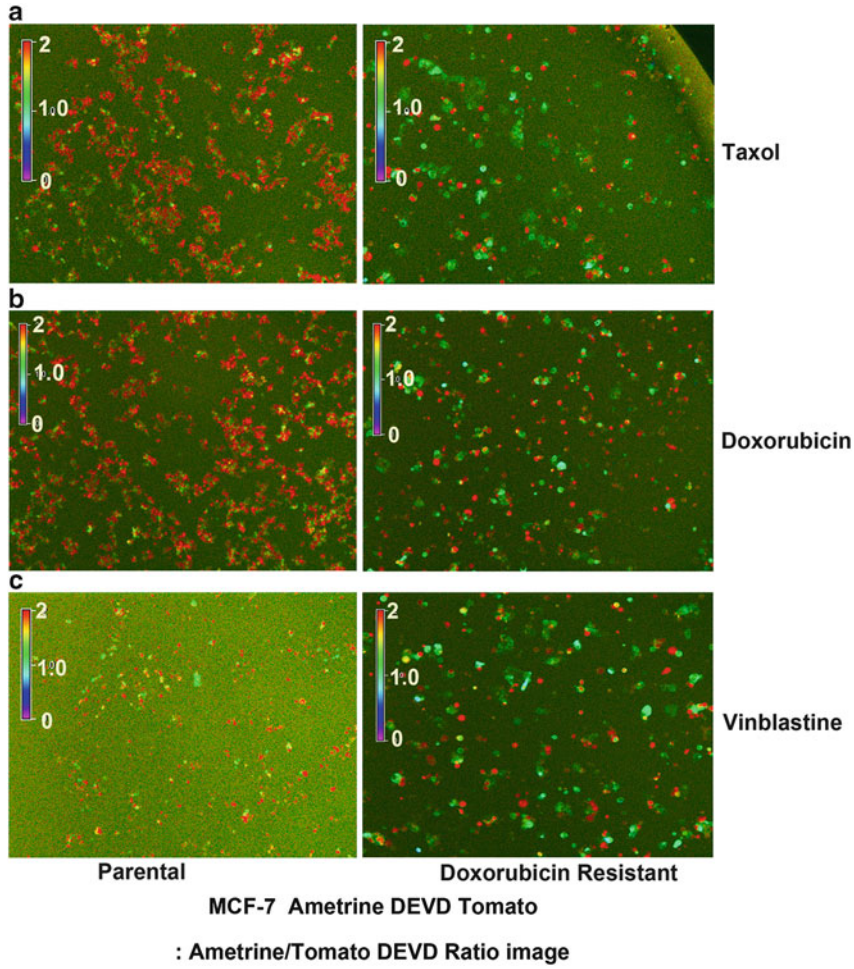

Fig. 2.5 *Apoptosis resistance in drug-escaped cells.* MCF-7 Ametrine–DEVD–Tomato NES-stable cells were treated with doxorubicin for three cycles as described. The drug-escaped cells were further treated with Taxol (**a**), doxorubicin (**b**) and vinblastine (**c**) for 48 h. The ratio of pmAmetrine and tdTomato is shown. The parental cells were also treated with the same drug and shown for comparison

population maintenance. Another emerging concept of tumour stem cells also supports the tumour recurrence after chemotherapy, emphasising that a small fraction of cells within a tumour is responsible for the tumour initiation, and they are resistant than the bulk tumour cells. This hypothesis explains the immediate response to drugs as revealed by the death of the bulk cells; however, the small fraction remaining after chemotherapy reinitiates the tumour later. Previously, several studies have reported the generation of drug-resistant cells with or without stem cell characteristics after initial drug treatment. Sharma et al. (2010) showed induction of reversible drug-tolerant state in non-small cell lung cancer cells to a lethal exposure of EGFR–TKI (Sharma et al. 2010). They also observed that the drug-tolerant cells were enriched with putative progenitor marker CD133. Similarly, we have recently employed an in vitro model system where breast cancer cell lines were exposed to diverse antitumour agents that generated drug-tolerant cells. We have employed breast cancer cell lines expressing live cell caspase detection FRET probe involving ECFP–EYFP linked with DEVD sequence, a preferred site for activated

caspases. This system helped us to visualise and track the fate of these cells for a long time using high-throughput imaging. Even though the system substantiated the immediate death of the cell by caspase activation, few cells that resisted the initial drug treatment remained sensitive to apoptosis by spontaneous caspase activation. Even after removal of the drug, most cells succumbed to cell death slowly upon entering into division or remained quiescent with replicative senescence. This senescent-dominating phase is followed by rare emergence of growing colonies that repopulate the tumour. Interestingly, our studies using the emerging colonies indicate that drug-escaped cells are enriched with drug-resistant cells, drug efflux side population cells and cells with low reactive oxygen species subsequent to reactivation of antioxidant systems. Here, we further provide evidence that drug escape is general in nature and not restricted only to breast cancer cells. The colon cancer cells treated with multiple drugs also generate drug-tolerant cells that are enriched in tumour stem cell-like cells. Here, we also provide evidence for rare entry of senescent cells to cell cycle using the sensitive live cell cycle indicator FUCCI in SW480 cells. Even though the molecular switch that drives senescent cells to enter into cell cycle is not clear, it plays a critical role in the emergence of drug-tolerant cells both in breast and colon cancer cells. In this report, we have used a highly sensitive cellular system of live cell caspase activation Ametrine–DEVD–Tomato. Ratio imaging by microscopy utilising these cells is a powerful and sensitive tool for drug screening that is also adaptable for high-throughput image-based systems. Since our earlier studies substantiate that the drug-escaped cells are highly enriched with tumour stem cell markers like CD133, OCT4 expression, low ROS and high-invasion potential, they are the ideal cell source for identification of compounds that specifically target them. Even though tumour stem cells are now considered as the best target for drug development, because of their rarity and difficulty to characterise, it remains as a challenge for drug screening. Since the cell system employed here expresses a sensitive probe for caspase activation and is also enriched with drug-resistant tumour stem cell-like cells, they will form better tools for drug screening in future.

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