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Cancer stem cells and escape from drug-induced premature senescence in human lung tumor cells

Implications for drug resistance and in vitro drug screening models

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Keywords: cancer stem cell, lung cancer, antitumor drug, DNA damage, senescence, DNA topoisomerase II

In this study, using an in vitro human tumor model, we show that non-small lung adenocarcinoma A549 cells after treatment with DNA damaging antitumor drugs become permanently growth-arrested as a result of so-called drug-induced premature senescence (pseudo-senescence). However, a small fraction of drug-treated cells escapes pseudo-senescence that leads to re-growth of tumor cell population after drug treatment. We show that this re-growth is associated with the presence of cancer stem cells (CSCs) in lung tumor cell population. We also document that re-growth of CSCs can be greatly delayed if lung tumor cells are treated with drug/caffeine combination that leads to the inhibition of the ATM/ATR pathway and decreased phosphorylation of PKB/Akt at Ser473. We show that in non-treated A549 cells caffeine by itself induces a reversible growth arrest that is associated with increased fraction of so-called side population cells, containing CSCs. These results point to the existence of an unknown, caffeine-sensitive mechanism that controls the number of CSCs in lung tumor cell population. Full characterization of this mechanism may lead to the development of innovative cancer therapies, which are based on small molecular weight inhibitors of CSC differentiation and self-renewal, which mimic caffeine action. Our results have also important implications for drug screening tumor models in vitro.

Introduction

Anticancer therapy is still only partially effective, mainly due to inherent or drug-induced resistance of tumor cells to standard chemotherapeutics and radiotherapy. Resistance mechanisms observed in tumor cells include increased activity of the ABC transporters and changed response to anticancer treatment, including de-regulated cell death pathways. One of the tumor types that is inherently very resistant to anticancer therapy is lung cancer, currently the most common cause of cancer related death in both men and women. About 80% of all lung cancers is non-small cell lung cancer and the majority of these tumors, especially adenocarcinomas, has functional p53 pathway.[1]

Until recently, it was well established that most if not all antitumor agents exert their cytotoxic and antitumor activity by inducing apoptosis or other types of cell death in tumor cells. However, more recent data show that exposure to low doses of different antitumor agents leads to growth arrest of tumor cells, without signs of cell death, where cells acquire morphological and biochemical features of senescent cells.[2-8] This process resembles replicative senescence,[9] but is induced primarily in tumor cells with functional p53 and is termed drug-induced premature senescence (DIPS) or pseudo-senescence (reviewed in ref. 10). Premature senescence has also been observed in normal cells in vitro treated with DNA damaging agents.[11-13] More importantly, mice-bearing tumors treated with cyclophosphamide and lung tumors from patients following chemotherapy also show signs of pseudo-senescence, what suggests that this phenomenon may contribute to chemotherapy treatment outcome in vivo.[8,14,15] For this reason, induction of pseudo-senescence by chemotherapeutic agents has been proposed to constitute a new strategy in anticancer treatment, that leads to irreversible proliferation arrest of tumor cells instead of induction of cell death. It should be stressed, that the therapeutic potential of premature senescence induction by anticancer treatment strongly relies on the irreversibility of this process and on the fact that it is induced in all tumor cells.

It is currently well established that many tumor types, including lung cancer, contain a small fraction of cells, with stem cell-like properties (called cancer stem cells, CSCs), that appear to be resistant to apoptosis induced by therapeutic agents (reviewed in refs. 16–20). It seems that CSCs possess two apparently contradictory features i.e., must be mostly non-proliferating cells as other stem cells are. At the same time, they must also be cycling cells to explain their persistence in the tumor and in cell culture. For that reason, the term of cancer stemloids has been recently

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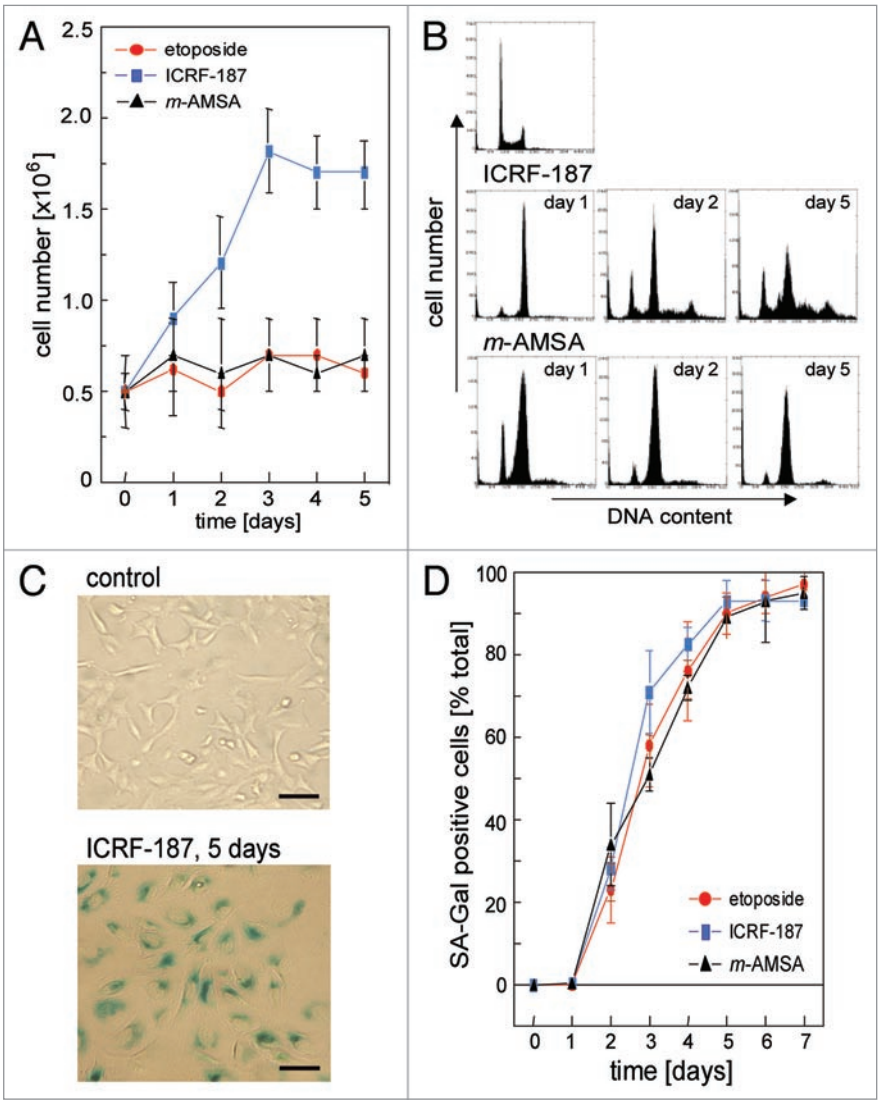

Figure 1. Induction of drug-induced premature senescence in A549 cells by studied drugs. Changes in cell number (A) and cell cycle distribution (B) during treatment with the IC_{80} doses of etoposide, ICRF-187 and amsacrine; (C) SA-β-galactosidase staining in non-treated and ICRF-187-treated cells for 120 h (bar, 20 μm); (D) fractions of cells with increased SA-β-galactosidase activity (relative to total cell number) during treatment with studied drugs.

introduced, that adds stem-like cancer cells (stemloids) to the stem cell hierarchy, which have the ability to self-renew but also undergo active proliferation.[21] The presence of CSCs or cancer stemloids may explain why a standard anticancer treatment, that successfully eliminates only differentiated cancer cells, does not lead to cancer cure. It was shown that normal stem cells after treatment with DNA damaging agents enter prematurely cellular senescence.[22,23] In addition, earlier studies have shown that embryonic stem cells as well as embryonic carcinoma cells are very sensitive to genotoxic stress induced by γ-irradiation and DNA damaging drugs.[24,25] If this was confirmed for cancer stem cells or stemloids, induction of premature senescence in tumor cell population by DNA damaging agents would turn a very effective anticancer strategy. However, this issue has never been explored experimentally.

In this study, using a well-characterized in vitro lung tumor model, non-small lung adenocarcinoma A549 cells, we wanted to clarify whether all cells from tumor cell population, including CSC cells, undergo pseudo-senescence induced by DNA damaging drugs. This is particularly important for a recently proposed anticancer strategy based on the induction of cellular senescence in tumor cells by anticancer agents. Human A549 cells retain typical features of lung cancer cells, such as aggressive phenotype and inherent resistance to radio- and chemotherapy. These cells possess a relatively high fraction of so-called side population (SP) cells, that has been reported to be enriched with CSCs.[26] For our studies, we selected a clinically important group of antitumor drugs, DNA topoisomerase II inhibitors, with various mechanisms of action toward the enzyme, both classical inhibitors (etoposide and *m*-AMSA or amsacrine) and the catalytic inhibitor (compound ICRF-187). In tumor cells, all these drugs induce direct and/or indirect DNA damage (reviewed in ref. 8). All types of non-small lung cancer cells overexpress topoisomerase IIα,[27] a primary target of the majority of topoisomerase II inhibitors. Moreover, one of the studied topoisomerase II inhibitors, etoposide, is a clinically used antitumor drug to treat lung cancer patients.

Results

Cells treated with topoisomerase II inhibitors enter long-term growth arrest with morphological and biochemical features of cellular senescence. Cells exposed to sublethal doses (corresponding to ~EC_{80} concentrations) of studied drugs, as determined by the MTT assay (not shown), ceased almost immediately cell proliferation (within 24 h) for etoposide and amsacrine or continued cell growth for 3 days for ICRF-187. Accordingly, cell number remained essentially unchanged or almost doubled during first 3 days of drug treatment and remained constant for next 2 days, suggesting that drug-treated cells entered long-term growth arrest (**Fig. 1A**). Majority of cells (>95%) exposed to ICRF-187 after 24 h arrested in G_2/M and at longer incubation times cells progressed to G_1 and polyploidy (**Fig. 1B**). For etoposide (not shown) and amsacrine (**Fig. 1B**), almost all drug-treated cells accumulated in G_2/M within 24 h, and a fraction of drug-treated cells (about 20%) entered polyploidy after longer incubation times.

We observed accumulation of cells with increased activity of SA-β-Gal (**Fig. 1C**), a broadly used biochemical marker of

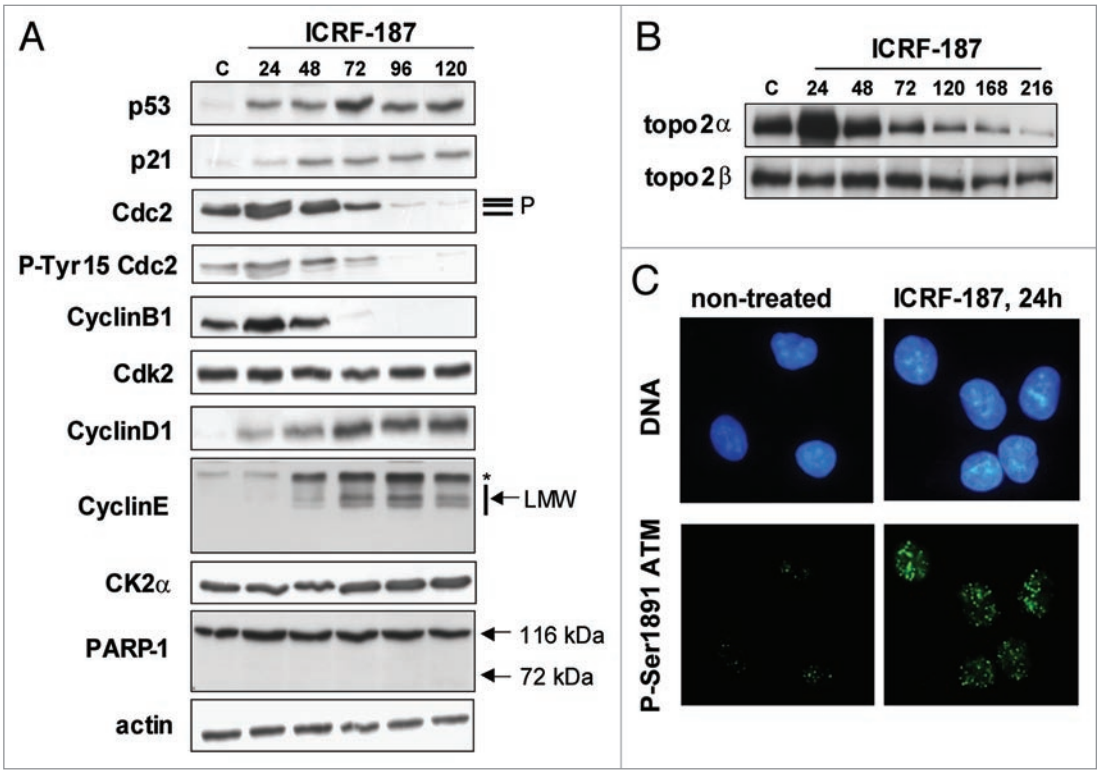

Figure 2. Activation of the DNA damage checkpoint in A549 cells treated with ICRF-187. (A) intracellular levels of cell cycle regulators, markers of cellular senescence and cell death; (B) changes in the intracellular levels of DNA topoisomerase II isoforms; (C) activation of the ATM kinase in non-treated cells and cells treated with ICRF-187, as revealed by immunofluorescence staining with anti-phospho-ATM specific antibody (green) and analysis by fluorescence microscopy. Cells were counterstained with DAPI (blue) to show nuclear morphology. P, phosphorylated forms of Cdk1; asterics shows the position of cyclin E and LMW low molecular weight forms of cyclin E, respectively.

cellular senescence.[28] The kinetics was very similar for all studied topoisomerase II inhibitors, and more than 95% of all cells were SA-β-Gal positive after 120 h of drug treatment (**Fig. 1D**). Drug-treated A549 cells become large with flat morphology (**Suppl. Fig. 1A**) and greatly expanded lysosomal compartment (**Suppl. Fig. 1B**). All these morphological and biochemical features were indicative of cells undergoing premature cellular senescence.

Drug-induced growth arrest is associated with the activation of DNA damage checkpoint and specific gene expression profiles. We also characterized changes in the expression of important regulators of cell proliferation and markers of apoptosis in drug-treated cells. Exposure to all studied drugs led to induction of DNA damage response in A549 cells, as exemplified for cells treated with ICRF-187, with a progressive increase of p53 and p21 levels and accumulation of phosphorylated forms of Cdk1 and cyclin B1 after 24–48 h of drug treatment (**Fig. 2A**), followed by downregulation of both proteins to undetectable levels at 120 h. This was indicative for the activation of DNA damage response in drug-treated A549 cells. In contrast, intracellular levels of other cell cycle regulators were either unchanged (Cdk2) or upregulated (cyclins D1 and E).

Decreased expression of the alpha subunit of casein kinase 2 was proposed as a molecular marker of cellular senescence.[29] However, no change in CK2α levels in A549 cells treated with ICRF-187 was observed (**Fig. 2A**). No cleavage of PARP-1 was detected, a well-known substrate of execution caspases, and a gradual decrease in the expression of topoisomerase IIα but not the isoform beta of this enzyme (**Fig. 2A and B**). Very similar pattern of protein expression was observed in for other drugs (not shown). Activation of the DNA damage checkpoint in drug-treated A549 cells was further confirmed by immunofluorescence staining for the activated ATM kinase. Phosphorylation of ATM at serine 1981 was very low in non-treated cells but greatly increased in cells treated with ICRF-187 (**Fig. 2C**) and other studied drugs (not shown).

Post-incubation of drug-treated cells leads to re-growth of a small fraction of cells. Previous studies suggested that induction of pseudo-senescence by DNA damage leads to irreversible growth arrest.[2-8,12,14] To determine whether proliferation arrest induced by studied drugs in A549 cells is irreversible, we first exposed these cells to studied drugs for 120 h and post-incubated in drug-free medium. After 2–3 days of post-incubation of cells treated with ICRF-187, amsacrine (**Fig. 3A**) and etoposide (**Suppl. Fig. 2**) a gradual increase in cell number was observed. Flow cytometry analysis revealed that ICRF-187-treated cells, which initially arrested in G_2/M, with an important fraction of polyploid cells, re-started cell proliferation during post-incubation and after 10 days cell cycle distribution was similar to that observed in non-treated cell population (**Fig. 3B**). Similar results were obtained for amsacrine and etoposide (not shown). We determined the

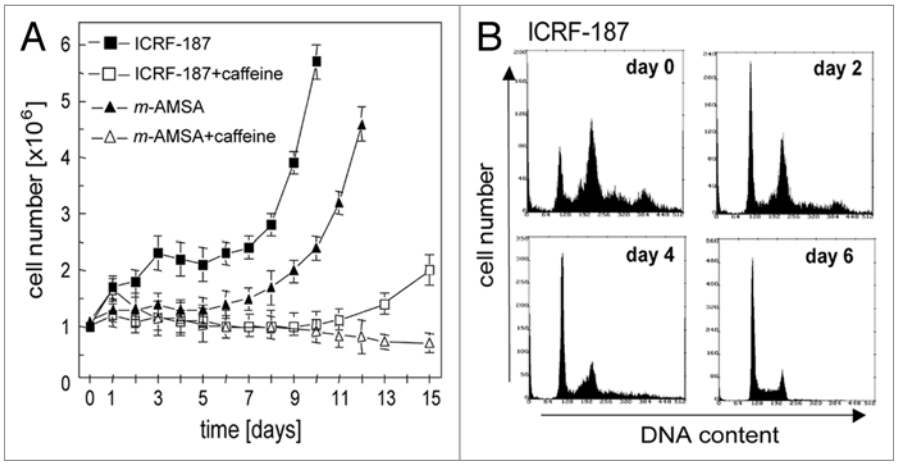

Figure 3. Escape from drug-induced premature senescence after treatment with studied drugs. Cell were treated with ICRF-187 and amsacrine in the absence or presence of 1 mM caffeine and post-incubated in drug-free medium. (A) growth curves for cells treated in the absence (empty symbols) and presence of caffeine (full symbols); (B) changes in cell cycle distribution of cells treated with ICRF and caffeine for 120 h and post-incubated for the time indicated.

fraction of cells that was able to re-grow after drug treatment by colony formation assay and for all studied drugs this fraction amounted to about 1–3% of the total cell population (**Suppl. Table 1**).

We expanded several different cell clones from amsacrine- and ICRF-treated cell populations, which escaped pseudo-senescence and established stable cell lines, designated A1910 and A0911, respectively. Interestingly, these cells had unchanged sensitivity to all studied drugs (**Suppl. Table 2**) and were still able to undergo growth arrest following treatment with studied drugs, with pseudo-senescence features and similar changes in protein expression during drug treatment (not shown).

Cells that escape pseudo-senescence after drug treatment originate from SP and CSC fractions. We hypothesized that A549 cells, which escape drug-induced pseudo-senescence, might be associated with the SP fraction and possibly CSCs. The SP fraction in non-treated A549 cell population amounted to about 10% of all cells but completely disappeared when A549 cells were stained with compound Hoechst 33342 in the presence of fumitremorgin C (**Fig. 4A**) or reserpine (not shown), inhibitors of ABCG2 and ABCC1/3 transporters, respectively. Treatment of A549 cells with ICRF-187 or two other drugs (**Fig. 4A** and **Suppl. Table 1**) greatly decreased SP fraction. More importantly, staining of the SP fraction with Hoechst 33342 after drug treatment was insensitive to both fumitremorgin C (**Fig. 4A**, bottom right graph) and reserpine (not shown). It follows that drug treatment of A549 cells does not lead to selection of cells that overexpress ABC transporters.

To confirm that SP sub-population in non-treated A549 cells is enriched in CSC fraction and we stained these cells for several different stem cell markers. Our results show that a small fraction of A549 cells was stained positive for CD34 (2.3 ± 0.25%) and CD117 (c-kit) (2.7 ± 0.15%), two typical markers of stem cells.[16,17] A fraction of A549 cells (1.1 ± 0.20%) was stained positive for both CD34 and CD117 (**Fig. 4B**). The fraction of cells expressing CSC markers was quantitatively very similar to the fraction of cells escaping pseudo-senescence after drug treatment (**Suppl. Table 1**). It strongly suggested that these cells originate from the CSC fraction. Interestingly, cells from colonies formed 7 days after of drug treatment had about 3-fold increased SP fraction (**Fig. 4C**). Longer post-incubation of these cells without drugs (e.g., after treatment with ICRF-187 or other drugs, A1910 cell line) led to cell populations with SP fractions similar to that observed in non-treated A549 cells (**Fig. 4C**, and not shown).

Inhibition of the DNA damage pathway leads to a substantially prolonged drug-induced growth arrest. We observed that drug treatment of A549 cells leads to the activation of the DNA damage checkpoint response (**Fig. 2C**). We next wanted to clarify whether pharmacological modulation of drug-induced DNA damage response influences the ability of A549 cells, including SP cells and CSCs, to undergo proliferation arrest and pseudo-senescence after drug treatment. To this end, we tested several different inhibitors of the DNA damage response pathway and the ATM/ATR signaling such as caffeine, the Chk1 inhibitor—compound UCN-01 and PI3K kinase inhibitor—wortmannin. Both wortmannin and UCN-01 were very toxic to A549 cells at kinase inhibitory concentrations (not shown), especially after long incubation times (>24 h). In contrast, combined treatment with ICRF-187 or amsacrine and a non-toxic dose of caffeine (1 mM) led to a substantially prolonged inhibition of cell growth. As shown in **Figure 3A**, no increase in cell number in drug-treated cell populations was observed for 5–10 days after drug treatment. Similar effect was observed for etoposide (**Suppl. Fig. 2**). Caffeine did not change the overall response to studied drugs and A549 cells treated with drug/caffeine combinations had morphological and biochemical features of senescent cells, with very similar kinetics to that observed for cells treated with studied drugs alone, including increased activity of SA-β-galactosidase (**Suppl. Fig. 3A**). Cells treated with both caffeine and ICRF-187 or two other drugs arrested primarily in G_2/M with very low polyploid fraction (**Suppl. Fig. 3B**, and not shown). In addition, there was no increase of p53 levels in drug-treated cells but

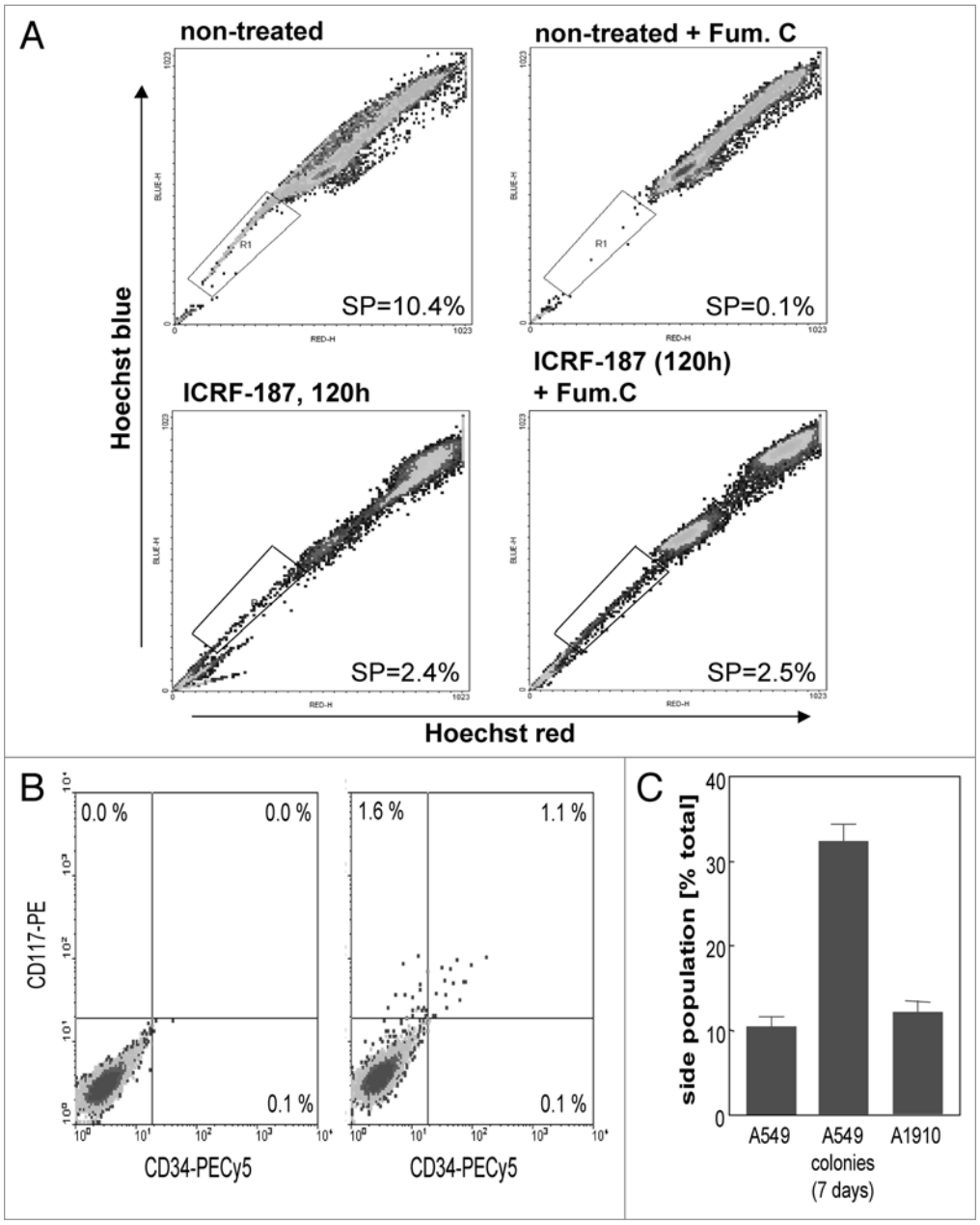

Figure 4. Side population and cells with stem markers in non-treated and ICRF-187-treated A549 cells. (A) non-treated cells or cells treated with the drug for 120 h were analysed for SP content. (B and C) Stem cell markers (B) and SP fractions (C) in non-treated A549 cells, cells escaping pseudo-senescence after treatment with ICRF-187 after 7 days (colonies) and after about 40 doublings after drug treatment (A1910). Live cells were stained for stem cell markers or with Hoechst 33342 with or without Fumitremorgin C and analyzed by two-parameter flow cytometry as described in Materials and Methods. Representative density plots or mean values of three independent experiments are shown.

expression of p21 was still induced as was a rapid downregulation of both Cdk1 and cyclin B1 (**Fig. 5A**). Interestingly, the fraction of cells that were able to re-start cell proliferation after drug treatment remained unchanged and still amounted to 1–2% of total cell population but occurred 5 to 10 days later (**Fig. 3A**).

Caffeine prevents phosphorylation of Akt/PKB kinase mediated by ATM/ATR. We then asked the question about the molecular mechanism responsible for the prolonged growth arrest of A549 cells induced by drug-caffeine combination. We observed only partial suppression by 1 mM caffeine of ATM activation in drug-treated cells, as revealed by immunofluorescence staining for phospho-Ser1981 ATM (not shown). Similarly, phosphorylation of p53 at Ser15 by ATM/ATR kinases remained essentially unchanged in cells treated with ICRF-187 or other drugs in the presence of caffeine (**Fig. 5B**, and not shown). This suggested that, in addition to ATM/ATR kinases, other signaling pathways could be also affected by caffeine.

One of the in vitro targets of caffeine is mTOR kinase, that activates and phosphorylates Akt/PKB kinase at Ser473.[30,31] To elucidate whether mTOR is inhibited by caffeine in A549 cells,

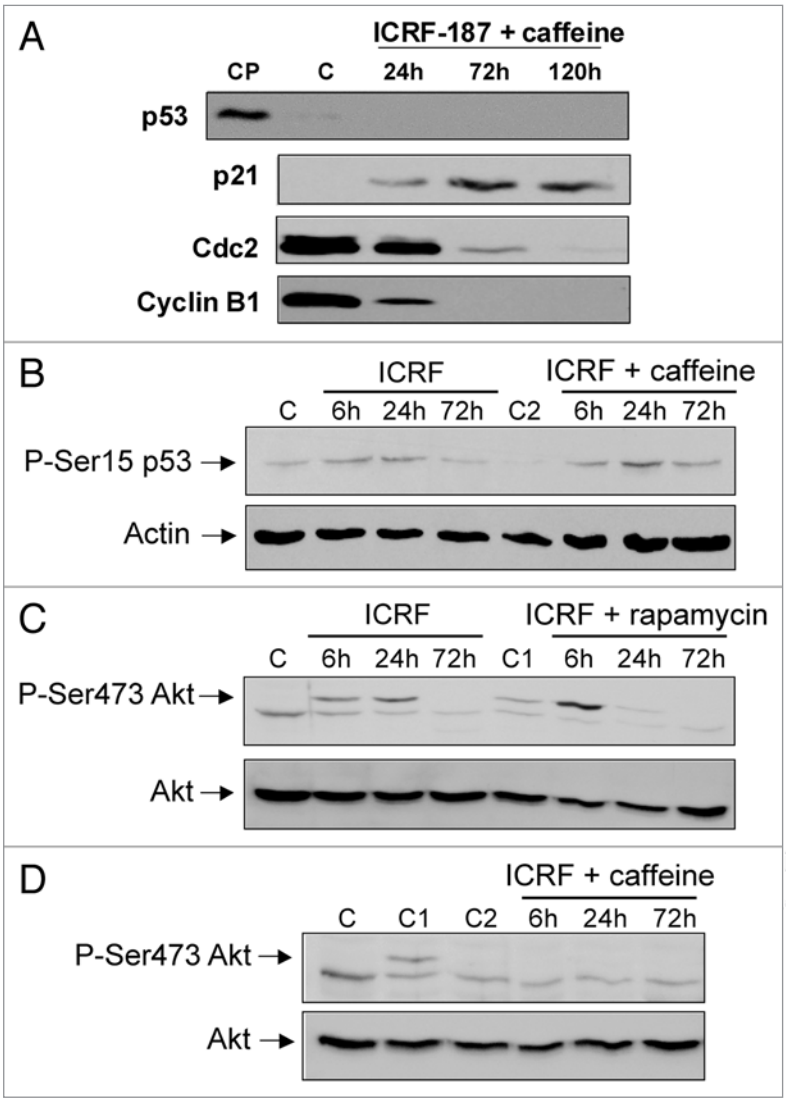

Figure 5. Activation of PKB/Akt in A549 cells treated with 75 μM ICRF-187 in the presence of 1 mM caffeine or 10 nM rapamycin for the time indicated. (A) expression of p53 and regulators of G_2/M transition; (B) phosphorylation of Ser15 of p53; (C and D) activation of Akt by phosphorylation at Ser473. (C) non-treated cells; CP, lysates from cells treated with 0.16 μM m-AMSA for 120 h, which serve as a positive control for increased p53 levels; C1 and C2, lysates from cells treated with 10 nM rapamycin or 1 mM caffeine for 24 h, respectively.

we followed the phosphorylation status of Akt kinase at Ser 473 by western blotting. Treatment of cells with ICRF-187 induced a transient Ser473 phosphorylation (**Fig. 5C**), however, activation of Akt/PKB was completely abrogated in the presence of caffeine (**Fig. 5D**). This effect was not associated with the inhibition of mTOR by caffeine as co-treatment of cells with ICRF-187 and rapamycin, a specific inhibitor of mTOR complex, lead to increased rather than decreased phosphorylation of Akt/PKB at Ser 473 (**Fig. 5C**). Combined treatment of A549 cells with studied drugs and rapamycin led to the induction of pseudo-senescence and re-growth of 1–2% of cells with similar kinetics as that observed for cells treated with antitumor drugs alone (not shown).

Caffeine inhibits proliferation/differentiation of CSCs. Finally, we determined whether caffeine influences SP and CSC fractions both in non-treated and drug-treated A549 cell populations. At studied concentration, caffeine did not appreciably change the growth characteristics of A549 cells but it lead to a gradual increase of SP fraction up to >60% cells after 10 days (**Fig. 6A**). After longer incubation with caffeine (>14 days), the cells gradually ceased proliferation and >95% of cells arrested in G_1 phase (not shown). However, this growth arrest was fully reversible and cell re-started cell proliferation 2–3 days after caffeine removal (not shown). In contrast, combined treatment of cells with ICRF-187 and caffeine for 120 hours did not significantly change the fraction of CD117-positive cells as well as SP fraction (**Fig. 6B and C**). We only noted that a small fraction of A549 cells treated with drug/caffeine combination (about 0.5%) disappeared from the SP population in the presence of fumitremorgin B (**Fig. 6C**).

Discussion

In this study, using an in vitro lung tumor model we addressed the question whether lung cancer stem cells when treated with DNA damaging antitumor drugs irreversibly arrest their proliferation by induction of pseudo-senescence. This issue has never been experimentally explored and relatively little is known about the effect of DNA lesions on cancer stem cells and its relation to drug resistance. It was shown that stem cells after treatment with antitumor drugs prematurely enter cellular senescence.[22,23] Other studies have shown that embryonic stem cells as well as embryonic carcinoma cells are very sensitive to genotoxic stress induced by γ-irradiation and DNA damaging drugs.[22,23,25] If this was confirmed for cancer stem cells, a new antitumor strategy, based on the induction of irreversible proliferation arrest of tumor cells as a result of drug-induced premature senescence, would be very effective. On the other hand, it has been shown that normal stem cells have only partially active DNA damage checkpoints.[24,32,33] This suggested that CSCs can actually be less prone to undergo irreversible growth arrest after exposure to DNA damaging agents. This, in turn, would result in an ineffective treatment. In addition, during drug treatment CSCs may accumulate additional genetic mutations and can re-establish tumors with more aggressive phenotype and highly resistant to anticancer treatment.

Our results show that after treatment with DNA damaging antitumor drugs, the majority of lung adenocarcinoma A549 cells become growth-arrested due to the induction of pseudo-senescence. These senescent A549 cells had all the morphological, biochemical and molecular markers of senescent cells, including flat morphology and increased activity of SA-β-galactosidase. Analysis of protein expression profiles in drug-treated cells

showed senescence-specific downregulation of cyclin B1 and Cdk1/cdc2 expression as well as upregulation of p21 and cyclin D1 levels. This phenotype was also observed previously by other groups in senescent tumor cells after chemotherapy.[2-8,14,15] It also supports the hypothesis proposed recently that the molecular mechanism of cellular senescence involves activation of growth-stimulatory signaling in cell cycle-arrested cells.[34]

We show that a small fraction of A549 cells (about 2%) escapes premature senescence after drug treatment and these cells have CSC features. Several other reports showed previously that treatment of different tumor cells with antitumor drugs leads to premature senescence but infrequent cells evade this process and re-enter proliferation.[6-8] Evasion from drug-induced premature senescence has been attributed to the ability of at least some tumor cells to upregulate Cdk1/cdc2 levels and activity.[7,8] In our studies, we did not observe any changes in the expression of Cdk1 in A549 cells which escaped premature senescence induced by studied drugs. More importantly, these cells were still able to undergo premature senescence after treatment with the same drugs and to downregulate Cdk1 expression. Moreover, A549 cells, which do not undergo pseudo-senescence after drug treatment, produce tumor cell populations with unchanged sensitivity to studied drugs. This is contrast to previous reports about the drug resistance phenotype observed in breast tumor MCF-7 cells that escaped doxorubicin-induced senescence.[7]

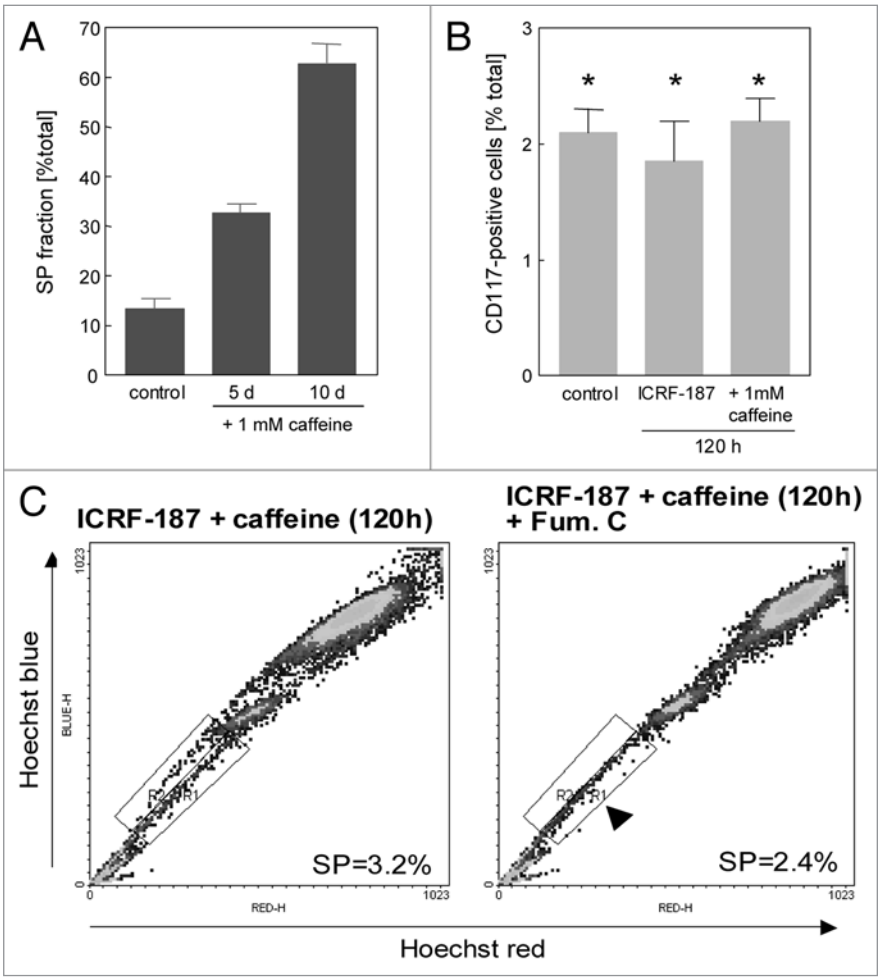

Figure 6. Effect of caffeine on the SP fraction in non-treated A549 cells (A), expression of CD117 (B) and SP fractions in cells treated with ICRF-187 in the absence or presence of 1 mM caffeine for 120 h (C). Representative density plots or mean values of three independent experiments are shown. Asterics, differences are statistically not different (Student t-test, $p < 0.05$).

Intriguingly, inhibition of the ATM/ATR pathway and phosphorylation of Akt at Ser473 during drug treatment of A549 cells substantially prolonged the time required for re-growth of lung tumor cells. Caffeine did not change an overall cellular response to studied drugs but inhibited proliferation and/or differentiation of the SP cells and CSCs. In agreement, previous studies showed that self-renewal of stem cells depends on the functionality of the ATM/ATR pathway and Akt/PKB activity.[35,36] Importantly, Akt/PKB kinase is one of the targets of both ATM and ATR kinases as well as DNA-PK.[37-39]

Caffeine has also been shown to inhibit mTOR kinase in vitro at low milimolar concentrations.[30] Moreover, recent reports showed that cellular senescence induced in immortalized epithelial cells by overexpression of p16 and p21, two inhibitors of cyclin-dependent kinases, may be decelerated by a specific mTOR inhibitor rapamycin.[40,41] However, inhibition of mTOR by rapamycin was ineffective in preventing irreversible growth arrest of epithelial cells induced by long-term exposure to two DNA damaging agents, doxorubicin and hydrogen peroxide.[40,41] Our results show that rapamycin is unable to suppress Ser473-Akt phosphorylation as well as to prevent re-growth of A549 cells after treatment with ICRF-187 and other studied drugs. In contrast, inhibition of mTOR by rapamycin led to a substantially increased Akt/PKB phosphorylation at Ser473 in non-stressed A549 cells, which was further potentiated by drug treatment. This effect may be attributed to the release of PI3K signaling from the negative feedback loop by inhibiting mTORC1 by rapamycin, that results in Akt/PKB activation.[31,42,43] Whatever the mechanism of the observed increased phosphorylation of Akt at S473 in drug-treated cells in the presence of rapamycin, these results clearly show that it is not mTOR inhibition by caffeine, at least its rapamycin-sensitive component mTORC1, that is involved in the delayed re-growth of cells in drug-treated cell population. It strongly supports the notion that the effect of caffeine on Akt/PKB phosphorylation/activation is mediated through the DNA damage response pathway.

Another important finding of our studies is that the number of SP and consequently CSC cells in tumor cell population in vitro is controlled by an unknown caffeine-sensitive mechanism. The nature of this mechanism is unclear but the number of the SP and CSC cells may depend on effects exerted by differentiated A549 cells (either non-treated or drug-treated cells) that may behave as a cell niche for CSCs.[44] For example, these differentiated lung tumor cells may secrete factor(s), which inhibit CSC self-renewal and/or differentiation of immature tumor initiating cells to fully mature lung tumor cells. If this mechanism was fully characterized and confirmed in other tumor models, this would lead to the development of innovative cancer therapies based on small molecular weight inhibitors of CSC differentiation and self-renewal, which can be successfully applied in cancer therapy to increase the efficacy of standard anticancer treatment.

Together, we show here that a new therapeutic strategy based on the induction of drug-induced premature senescence in tumor cells by antitumor drugs, as an alternative to apoptosis, cannot be fully efficacious toward lung tumor cells. This results from the fact that lung CSCs do not undergo an irreversible growth arrest after treatment with DNA damaging drugs and escape premature senescence. This leads to re-growth of tumor cell population after drug treatment. However, we show that re-growth of lung tumor CSCs after drug-treatment, can be greatly delayed by the combined treatment with DNA damaging drugs and caffeine, and this effect may be attributed to the inhibition of ATM/ATR-dependent phosphorylation of Akt/PKB at Ser473.

Our results have several important practical implications for anticancer treatment. First, we show a new direction in cancer therapy in which one could prevent or greatly delay the re-growth of tumor cells following drug treatment, solely by inhibiting CSCs differentiation/self-renewal, not CSCs killing. This effect may be produced by compounds that could mimic caffeine action, such as recently developed selective inhibitors of Akt/PKB or ATM.[45,46] One may also predict that such compounds will delay the appearance of fully developed tumor cells during carcinogenesis and therefore may be used in cancer chemoprevention. Secondly, induction of pseudo-senescence by DNA damaging drugs in large populations of lung carcinoma cells maintained in vitro may make also possible the selection of relatively unlimited numbers of cancer stem cells or stemloids, whose biology may then be studied with far greater facility in simple and well-controlled conditions. This is important given that mouse xenotransplantation systems, frequently used in cancer stem studies, are increasingly criticized.[47,48] Equally important, by generating sufficient amounts of lung cancer stem cells or stemloids, and possibly those present in other tumor types, we may be able to modify drug screening models to fine-tune existing protocols to more efficient targeting of CSCs/stemloids by new antitumor drugs or drug combinations. Finally, based on our results we propose a new drug screening procedure where tumor cells in vitro are exposed to different antitumor drugs and surviving cells are evaluated for the presence of cancer stem cell using e.g., membrane stem cell markers. This new screening system could replace the more traditional approach in which CSCs are first sorted from the bulk tumor cell population and then used in drug cytotoxicity/growth inhibition assays.

Methods

Drugs, antibodies and chemicals. Compound ICRF-187 (Cardioxane®) was from Chiron (The Netherlands), etoposide from Sigma-Aldrich-Fluka (Poznan, Poland), amsacrine (*m*-AMSA) was synthesized in our Department by Dr. Marek Konieczny while UCN-01 was kindly provided by Dr. Edward A. Sausville (University of Maryland, MD). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), propidium iodide, RNase A, caffeine and wortmannin were purchased from Sigma-Aldrich-Fluka, rapamycin was from Axxora (San Diego, CA). Cell culture media, antibiotics and serum were from Hyclone. Monoclonal mouse anti-p53 (clone DO-1), anti-Cdc2, polyclonal rabbit anti-cyclin B1, polyclonal goat anti-actin antibodies were from Santa Cruz, monoclonal PE-Cy5-conjugated anti-CD34, PE-conjugated anti-CD117 antibodies and isotype IgG-specific antibodies conjugated with respective fluorochromes were from BD Biosciences. Monoclonal mouse anti-phospho-Ser-15 p53, anti-phospho-Ser473 Akt as well as anti-Akt were from Cell Signaling, monoclonal mouse anti-phospho-Ser-1981 ATM from Upstate Biotechnology. Horseradish peroxidase-conjugated anti-mouse, anti-rabbit and anti-goat IgG antibodies as well as FITC-conjugated anti-mouse or anti-rabbit antibodies were from Jackson ImmunoResearch Labs.

Cell lines. Human non-small lung carcinoma A549 cells were originally from ATCC and provided by Dr. Annette K. Larsen (Hôpital Saint-Antoine, Paris). A1910 and A0911 cells were obtained by exposure of parental A549 cells to amsacrine (0.16 μM) or ICRF-187 (75 μM) for 120 h and post-incubation in drug-free medium until proliferating cell clones appeared. Clones were randomly picked up and expanded. Cell doubling time, morphology and cell cycle distribution of A1910 and A0911 cells was comparable to that of the parental A549 cells. All cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). Cells were grown at 37°C in 5% CO_2/air atmosphere and screened routinely for Mycoplasma by the PCR method with Mycoplasma *Plus* PCR Primer Set (Stratagen).

Drug treatment. All drugs except ICRF-187 were dissolved in DMSO and used from 10 mM stock solutions. ICRF-187 solutions (100 mM) were freshly prepared in sterile water.

Cytotoxicity assay. Exponentially growing cells were exposed to the indicated drug concentrations for 5 days and the viability determined by the MTT assay. In some experiments, cells were exposed to drugs for 24–72 h, washed and post-incubated in the absence or presence of response modulators followed by post-incubation in drug-free media for up to 5 days. The IC_{80} values are defined as the drug concentration resulting in 80% reduction of viable cells, compared to untreated control cells.

Flow cytometry. The cell cycle distribution was determined by flow cytometry using a FACScan flow cytometer (Becton Dickinson) equipped with an argon laser to give 488 nm light as described earlier.[49] The percentage of cells in each phase of

the cell cycle was calculated using MultiPlus software (Phoenix Flow Systems).

Side population was determined according to published procedures.[26,50,51] In some experiments, cells were incubated with 5 μg/ml Hoechst 33342 in the presence of 50 μM reserpine or 10 μM fumitremorgin C. Cell samples were analyzed with a LSR II flow cytometer (Becton Dickinson) equipped with UV laser to give 355 nm excitation and respective filters for dual emission wavelength analysis at 405/30 nm (blue) and 670/40 nm (red). Non-viable cells were excluded from analysis based on propidium iodide exclusion.

Western blot analysis. Cells were lysed in RIPA buffer (150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl, pH 8) containing a protease inhibitor cocktail (Roche Diagnostics GmbH) and phosphatase inhibitors (50 mM sodium fluoride, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate) for 15 min on ice. Protein concentrations in cellular lysates were determined by the BCA assay (Pierce). Equal amounts (50 μg per lane) were loaded in Laemmli buffer, separated by the SDS-PAGE electrophoresis in polyacrylamide gels and transferred onto PVDF membranes (Pierce). After transfer, membranes were blocked in 5% non-fat milk in TBS buffer (10 mM Tris-HCl, 150 mM NaCl, pH 8) and washed in TBST buffer (TBS buffer containing 0.05% Tween-20). Membranes were incubated with primary antibodies diluted in TBST containing 0.5% bovine serum albumin for 1–6 h at room temperature or overnight at 4°C. After three washes in TBST, membranes were incubated with secondary antibodies diluted at 1:40,000 in TBST for 1 h at room temperature. Results were revealed by the ECL kit (Pierce) according to the manufacturer instructions.

Immunofluorescence staining and microscopy. Following drug exposure, cells were washed with PBS, fixed in 70% ethanol at -20°C, rehydrated in PBS and permeabilized with 0.1% Triton X-100 for 5 min on ice following staining with respective antibodies as described previously.[49] All images were collected by an OLYMPUS BX-60 microscope equipped with a digital DP-50 camera using ImagePro acquisition program (Media Cybernetics). Images were further digitally processed for contrast enhancement by Adobe Photoshop.

The percentage of senescent cells was calculated based on manual counting under light microscope (with low magnification 5X objective), where the number of SA-Gal positive and negative cells was determined in blind-labeled samples.

The percentage of cells that escaped drug-induced senescence was determined based on the results of clonogenic assay. Briefly, cells were treated with studied drugs for 120 h, trypsinized, counted and seeded in triplicates with different cell dilutions onto 60 mm Petri dishes. Following post-incubation in drug-free medium for 7–8 days, colonies were stained with Giemsa dye and counted. Cloning ability was calculated as a fraction of cells from the total cell number seeded per culture dish that was able to produce cell clones.

For membrane epitope staining, aliquots of cell suspension (1 x 10^6 cells) were incubated with PE-Cy5-conjugated anti-CD34 or PE-conjugated anti-CD117 antibodies for 30 minutes on ice and analyzed immediately using a FACScan flow cytometer (Becton Dickinson). Results were further quantitated with MultiGraph software (Phoenix Flow Systems). Non-specific fluorescence was normalized using control cells stained with isotype IgG-specific antibodies conjugated with respective fluorochromes.

Staining for cellular senescence associated markers. Cells were stained for SA-galactosidase (SA-β-Gal) as described.[28] Alternatively, following treatment live cells were stained for 30 minutes at 37°C with 100 nM LysoTracker® Red (Molecular Probes), counter-stained with 0.5 μM Hoechst 33342 for 5 minutes and analyzed under fluorescence microscope as described above.

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Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/SabiszCC8-19-Sup.pdf

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