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ORIGINAL ARTICLE

Enhancement of cisplatin sensitivity by NSC109268 in budding yeast and human cancer cells is associated with inhibition of S-phase progression

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Abstract
Purpose NSC109268 has been described previously as inhibitor of proteasomal degradation and of phosphatase 2Cα. In a yeast screen, we isolated NSC109268 as an agent altering sensitivity to DNA-damaging agents. We found that NSC109268 and the related compound NSC109272 enhance cellular sensitivity to cis- and transplatin but reduce sensitivity to nitrogen mustard. We explored if similar effects could be found in human cancer cells and if cell cycle analysis could hint at the underlying molecular mechanism.
Methods Haploid yeast cells were treated in suspension with platinum agents and nitrogen mustard alone or in combination with NSC compounds, and survival was measured by colony-formation assays. Sensitivity of ovarian and prostate cancer cells toward these treatments was evaluated using the MTS assay. Cell cycle progression was determined by flow cytometry.
Results The enhancement of cisplatin sensitivity by NSC109268 found in yeast was confirmed in cisplatin-sensitive and cisplatin-resistant human ovarian cancer lines and in prostate cancer cells. In yeast and in human carcinoma cells, a correlation of enhanced sensitivity with delaying S-phase progression was revealed.
Conclusion The known activities of NSC109268 as proteasome or phosphatase inhibitor could explain the phenotype of S-phase delay by assuming a higher initial DNA damage load, inhibition of DNA translesion synthesis or extended checkpoint arrest.

Keywords Cisplatin · Resistance · Ovarian cancer · Yeast · Proteasome inhibitor · S-phase

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Introduction

In the majority of cancers, effective chemotherapy requires a combination of agents. There is continued interest in discovering preferably non-toxic compounds that increase the therapeutic index of an established drug. In this study, we have isolated such a novel adjuvant candidate in a yeast screen and established its likely usefulness for improving cisplatin (CP) sensitivity in ovarian cancer cells.

Ovarian cancer is the eighth most common cancer and the fourth most frequent cause of cancer death in women. Following surgical debulking, chemotherapy with CP or its derivatives in combination with paclitaxel is the prevalent treatment. Unfortunately, 20% of ovarian cancers display initial CP resistance and an additional 65% will respond initially but develop resistance. Overall, the 5-year survival

of ovarian cancer is only 45%. Thus, the isolation of agents that enhance cellular CP sensitivity and possibly overcome resistance is an active area of research [1, 14].

Cisplatin predominantly induces DNA intrastrand crosslinks (mainly 1,2-d(GpG) adducts) as well as a small percentage of interstrand crosslinks [13]. Whereas many anticancer agents introduce DNA damage, it is frequently not straightforward to rationalize their mechanism. In most cases, a response system to DNA damage at the "caretaker" or "gatekeeper" level appears to be altered in cancer cells, which include DNA repair and checkpoint-related responses such as cell cycle arrest, senescence, apoptosis and other cell death pathways. The initial step of DNA damage recognition during the course of checkpoint activation involves crosstalk between several sensor proteins. In the yeast *Saccharomyces cerevisiae*, one of these is Rad17 (Rad1 in *Schizosaccharomyces pombe* and *Homo sapiens*). Rad17 participates together with proteins Mec3 and Ddc1 in formation of a PCNA-like clamp complex [19]. We previously reported that certain DNA-damaging agents induce an increased self-interaction of Rad17 [32]. Analysis of Rad17 point mutations indicated an important role of Rad17 homomeric interaction in checkpoint activation, possibly through some kind of signal amplification by higher-order complex formation. By application of the yeast two-hybrid system, this increased interaction can be converted into a growth response [31, 32]. The developed assay is unique since it does not detect an indirect consequence of DNA damage, such as lethality or growth inhibition, but it measures an activity of a given compound that may directly reflect possible anticancer drug efficacy—the capability to trigger an early event associated with checkpoint activation. This assay proved to be especially sensitive to topoisomerase I inhibitors of the camptothecin type [31].

Following an initial screening attempt for modifiers of the camptothecin effect, we focused on the influence of the isolated candidate NSC109268 on CP sensitivity. In contrast to other DNA-damaging agents, NSC10928 enhanced cellular CP sensitivity in yeast as well as in ovarian or prostate cancer cells, suggesting its possible use as an adjuvant in cancer chemotherapy.

Materials and methods

Chemicals

Cisplatin, transplatin, camptothecin and mechlorethamine hydrochloride (nitrogen mustard) were purchased from Sigma Chemicals, carboplatin from LC Laboratories. The diversity set of compounds, as well as NSC109268 and NSC109272, were provided by the Developmental Therapeutics Branch of the National Cancer Institute. NSC109268 was also custom-synthesized by Omm Scientific, Dallas, TX.

Survival analysis in yeast

Haploid wild-type *S. cerevisiae* strain BY4741 was grown to early logarithmic phase in rich medium [1% yeast extract/2% peptone/2% dextrose (YPD)] and exposed for 2 h at 30°C to the drugs in phosphate-buffered saline (PBS). The cell suspension was appropriately diluted, plated onto YPD plates and incubated at 30°C to allow formation of colonies. Data are expressed as ratio of titers of macrocolony-forming cells in drug-treated samples (N) versus control samples (N_0).

Cell viability measurement by MTS assay in mammalian cell lines

The colorimetric MTS cell proliferation assay (Promega) was used to determine possible synergism between NSC109268 and CP. CP-sensitive ovarian carcinoma cell line 2008, CP-resistant ovarian carcinoma cell line 2008/C13 (originally provided by Dr. Stephen Howell) and prostate carcinoma line PC3 were cultured in RPMI1640 (Fisher Scientific), supplemented with 5% FBS (Fisher Scientific). Approximately, 8×10^3 cells/well were plated onto 96-well dishes and allowed to attach overnight in growth medium. The following day, cells were rinsed twice in RPMI1640 and further incubated in serum-free medium for 24 h. Cells were then treated with CP and/or NSC109268 for 24 h. Negative controls involved the use of DMF (diluent for cisplatin) and DMSO (diluent for NSC109268).

To determine the number of surviving cells, cells were incubated in 333 μg/ml MTS, a tetrazolium salt, and 25 μM PMS, an electron-coupling reagent, according to the manufacturers' instructions. MTS is taken up by live cells, reduced by the dehydrogenase enzymes and released back into the culture medium as a yellow formazan product. The amount of formazan product, measured by absorbance at 490 nm, is directly proportional to the number of living cells in the culture. Thus, absorbance readings taken after 1 h were converted to cell numbers based on standard curves. Experiments were performed in triplicate wells and repeated three times.

Synergism between NSC109268 and CP was evaluated by the fractional product method [4] as applied by Ager and Haynes [2]. Survival probability and average number of lethal events per cell (H) at a given dose x are estimated as $-\ln\ S(x) = H(x)$, with $S = N_x/N_0$. In case of a two-agent combination, $-\ln\ S(x,\ y) = H(x) + H(y) + h(x,\ y)$, with $H(x)$ and $H(y)$ corresponding to lethal hits contributed by the agents independently and $h(x,\ y)$ corresponding to

additional lethal hits contributed by agent interaction. If $h(x, y)$ is positive, zero, or negative, the combined effect can be termed synergistic, additive or antagonistic, respectively.

Synchronization and flow cytometric DNA analysis of yeast

Wild-type yeast *S. cerevisiae*, BY4741, was inoculated in YPD overnight at 30°C. Cells at a concentration of 0.5×10^7 cells/ml were spun down and resuspended in YPD. To 5 ml of culture was added 50 µl of 1 mg/ml α factor (Genescript) for cell synchronization. After incubating for 90 min at 30°C, another 50 µl of α factor was added and the culture was incubated for 30 min. The culture was then resuspended in 1 ml PBS. Various doses of CP and NSC109268 were then added together with 25 µl of α factor, and cultures were treated for 1 h at 30°C with shaking. Cells were washed and resuspended in YPD. Sample preparation and propidium iodide staining have been described elsewhere [25]. Cellular fluorescence was measured in a FC500 Flow Cytometer (Beckman Coulter Corp., FL), and histograms were analyzed using CXP software. Experiments were repeated at least twice.

Flow cytometric DNA analysis of mammalian cell lines

The 2008/C13 CP-resistant ovarian carcinoma cell line was cultured in RPMI1640 supplemented with 5% FBS (Fisher Scientific) at 37°C (humidified atmosphere, containing 5% carbon dioxide).We plated 4×10^5 cells onto 60-mm petri dishes and allowed to adhere overnight. Drugs were administered to 50% confluent cells in medium (RPMI1640 + 5%FBS). After 24 or 48 h of incubation, samples were taken for processing. First, supernatants were collected to harvest any floating apoptotic cells. Dishes were washed with ice-cold PBS which was combined in the same tube. Second, attached cells were trypsinized and collected separately. Tubes were spun, supernatant was discarded and ice-cold PBS was added to each tube followed by 70% ethanol to fix the cells overnight. Fixed cells were combined, counted and a total of approximately 6×10^5 cells were labeled with DNA-staining solution (RNAse A, 2.5 µl of 20 mg/ml and propidium iodide, 5 µl of 1 mg/ml).

Results

NSC109268 enhances sensitivity of yeast to cis- and transplatin but not nitrogen mustard

The described two-hybrid assay based on DNA-damage-enhanced homomeric interaction of Rad17 [31, 32] was modified to screen for agents that reduce or enhance such checkpoint-associated interaction in haploid yeast. The reporter strain was spread on selection media containing camptothecin. Subsequently, a library of 1,900 compounds (the Diversity Set provided by the Developmental Therapeutics Branch of the National Cancer Institute) was transferred to the plate using a replicating tool. Unusual density or absence of colony formations in the vicinity of a substance was identified. In the case of the organic copper compound NSC109268 (Fig. 1), we found an inhibition of colony formation, indicating less induced Rad17 interaction and thus possibly less checkpoint activation (data not shown). Further studies confirmed that NSC109268 could alleviate growth inhibition and killing by camptothecin.

Surprisingly, a survey of various drug interactions indicated the opposite effect with CP, TP where lethal effects in logarithmic-phase yeast were enhanced by coincubation with NSC109268 and the related compound NSC109272 (Figs. 1, 2a, b). Closer inspection of quantitative survival curves revealed effects on colony survival that were more than additive, i.e. the effects could not be explained by a combination of independent effects of the two simultaneously administered drugs. Similar synergistic effects were seen if cells synchronized in G1, G2/M or S-phase were treated (Fig. 2e and data not shown). If observed on plate following overnight incubation, non-colony-forming cells are characterized by chain formation, indicating repeated attempts at cell division. This overall response pattern to CP was not altered by treatment with NSC109268 although frequency of colony formation was lowered (Fig. 2e).

Similar effects were confirmed for carboplatin (Fig. 2c). Although of different structure and relative frequency, DNA interstrand crosslinks are lesions common to platinum agents and nitrogen mustard (NM). In contrast to the platinum agents tested, however, the cellular resistance to NM was reduced rather than enhanced if co-incubated with NSC109268 (Fig. 2d).

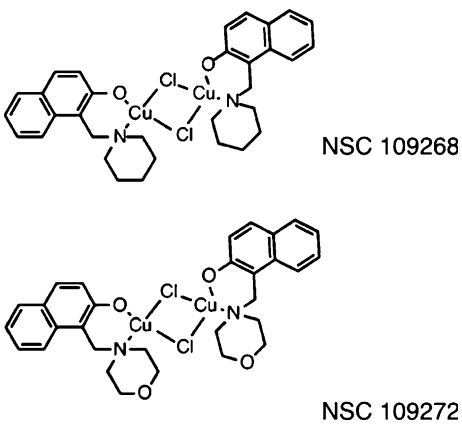

Fig. 1 Structure of NSC109268 and NSC109272 compounds

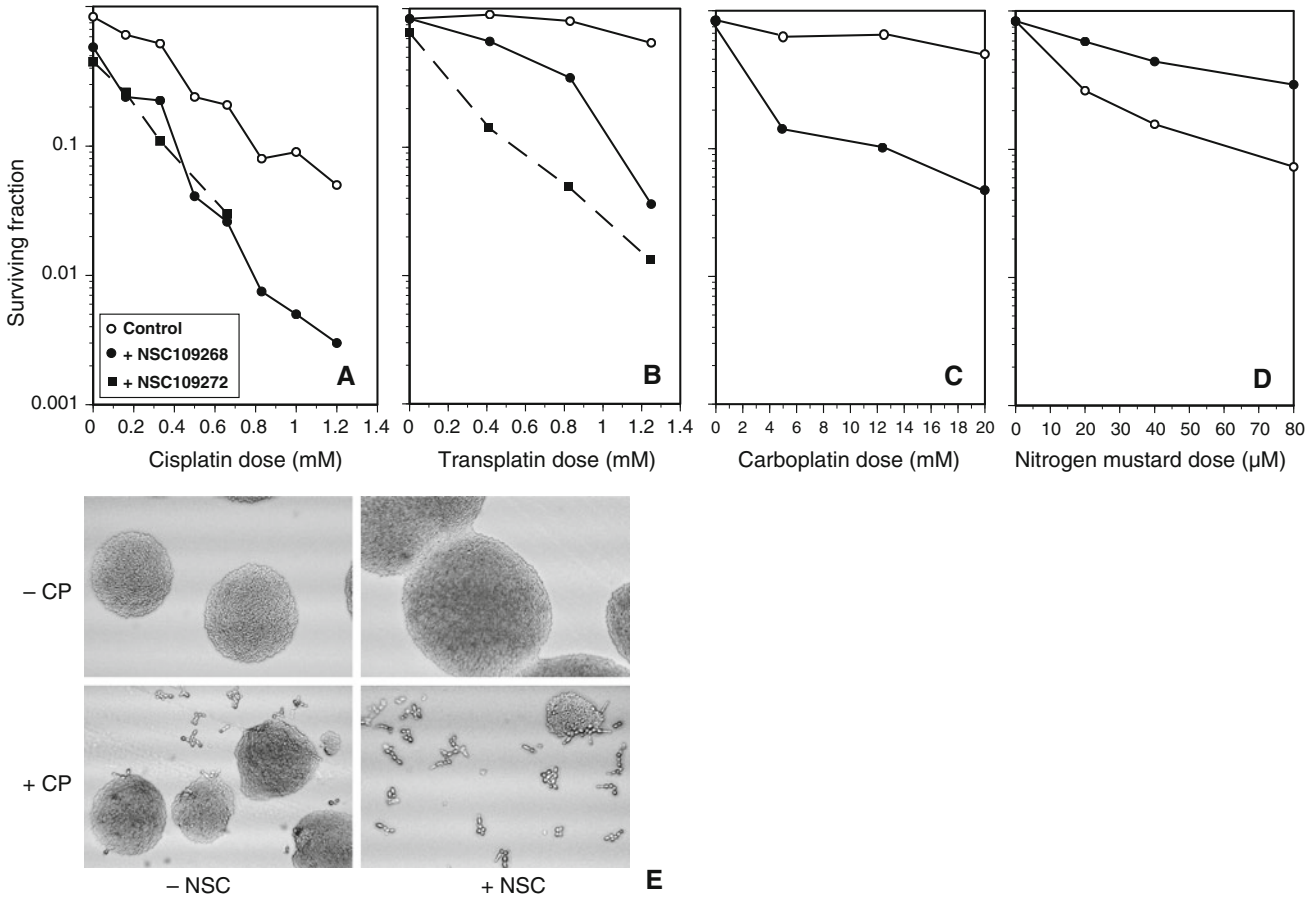

Fig. 2 Sensitivity of logarithmic-phase haploid yeast (BY4741) toward crosslinking agents is influenced by NSC109268 and NSC109272. Fractions of colony-forming cells were plotted as a function of dose of **a** cisplatin with and without NSC109268 (22 μM) or NSC109272 (6 μM), **b** transplatin with and without NSC109268 (2.2 μM) or NSC109272 (6 μM), **c** carboplatin with and without NSC109268 (4.5 μM), **d** nitrogen mustard with and without NSC109268 (2.2 μM) present during treatment. Representative single experiments are shown. **e** View of microcolonies and inactivated cells on plate, 24 h following treatment of G1-synchronized cells with 1.5 mM CP alone (*lower left*) or in combination with NSC109268 (4.5 μM; *lower right*). The upper row shows mock-treated or NSC109268 only treated cells

NSC109268 delays S-phase traversal in cisplatin-treated yeast

Haploid yeast cells were synchronized in G1 using yeast mating factor α and treated with NM, CP or TP in the presence or absence of NSC109268. Following treatment, cells were released into fresh medium, and cell cycle progression was monitored using flow cytometry. Treatment with NSC109268 alone resulted in a slightly delayed G1/S progression (Fig. 3a and data not shown). With NM, extensive arrest with G1 DNA content was noted. Addition of NSC109268 during NM treatment, however, had no additional effect on cell cycle progression. Treatment with a CP dose of comparable toxicity (around 20% in combination with NSC109268) resulted in slow G1/S progression with was further slowed down if NSC109268 had been present during treatment (Fig. 3a), followed by extensive G2/M arrest. Similar effects were confirmed for higher doses of CP as well as for TP (Fig. 3a).

Bud emergence in yeast is a landmark of exit from G1, and we used this criterion to distinguish G1 from early S-phase arrest. If budding kinetics were analyzed for NSC10268, CP and NM (both at low doses), a very short delay was seen for treatment with NSC109268 alone (Fig. 3b). For NM, significant G1 arrest was observed that was not influenced by NSC109268. This was in contrast to CP where no G1 arrest was noted. These observations identify the G1/S accumulation seen in the flow cytometric profiles as mostly in G1 for NM and mostly in early S for CP. Only the latter appears to be influenced by NSC109268.

NSC109268 enhances sensitivity of cancer cells to cisplatin

We were interested if these observations can be extended to human cancer cells. Since ovarian cancer cells are CP

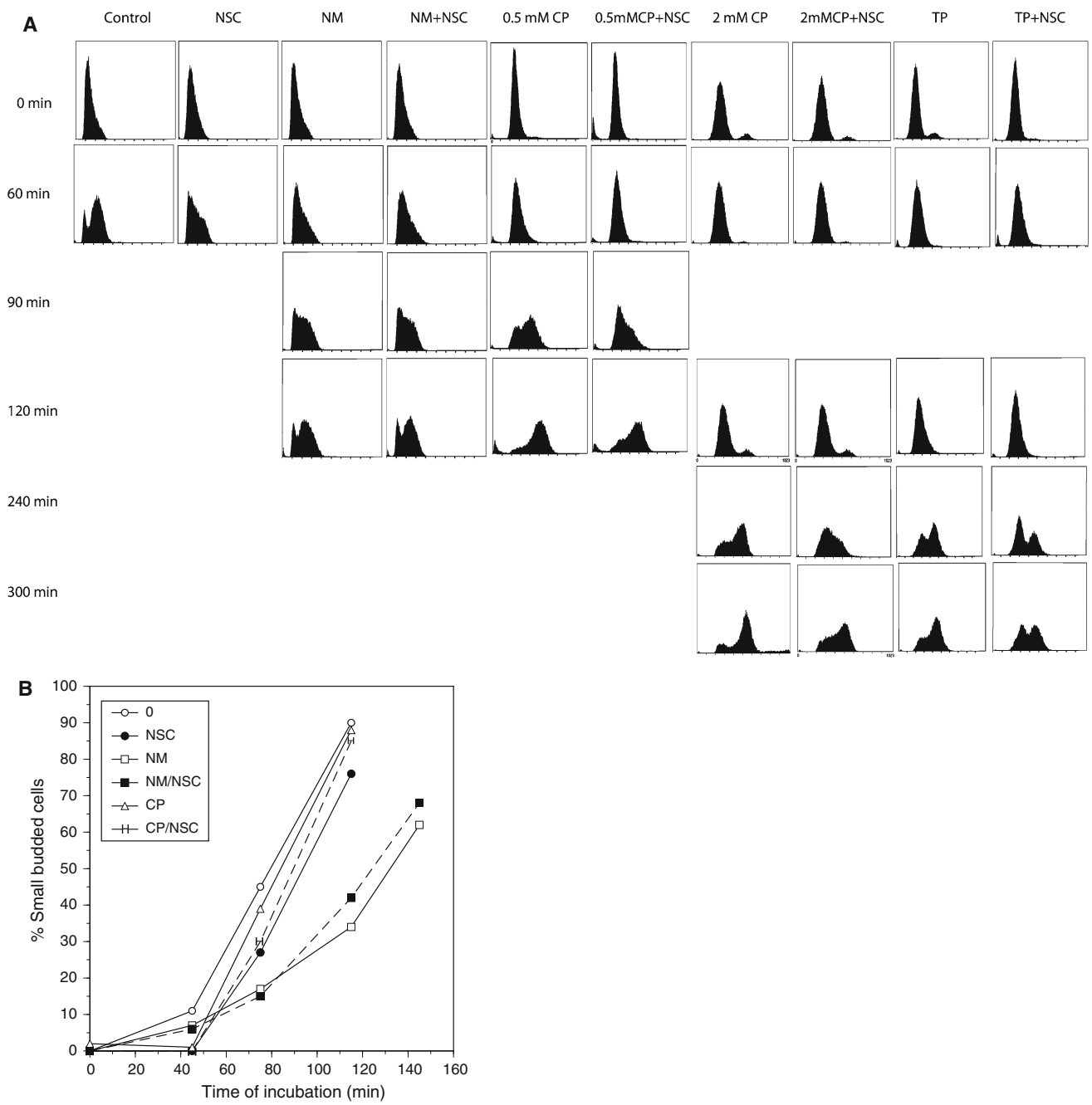

Fig. 3 Analysis of cell cycle progression following treatment of G1-synchronized yeast with crosslinking agents. **a** Flow cytometric analysis of DNA content following treatment with NSC109268 alone (4.5 μM) and in combination with NM (20 μM), CP (0.5 and 2 mM) and TP (1.2 mM). **b** Budding analysis following treatment with NM (20 μM) or CP (0.5 mM), without or in combination with NSC109268 (4.5 μM)

sensitive but acquisition of CP resistance by these cells is a significant problem in therapy, we examined if NSC109268 sensitizes these cells to CP. We used ovarian carcinoma line 2008 and its CP-resistant variant 2008/C13. In addition, we used an androgen-insensitive metastatic prostate cancer cell line (PC3). CP sensitivity was determined using various dose combinations with CP and NSC109268 in an MTS assay (Fig. 4a–c). The applied CP dose range reflects the known relative CP resistance of these lines. For the majority of combinations, more than additive sensitivity was evident for all three cell lines, as shown by mathematical analysis (Fig. 4d). Remarkably, 2008/C13 ovarian cancer cells appeared to be even more sensitive to NSC109268 alone and to combination treatments than the CP-sensitive parent line.

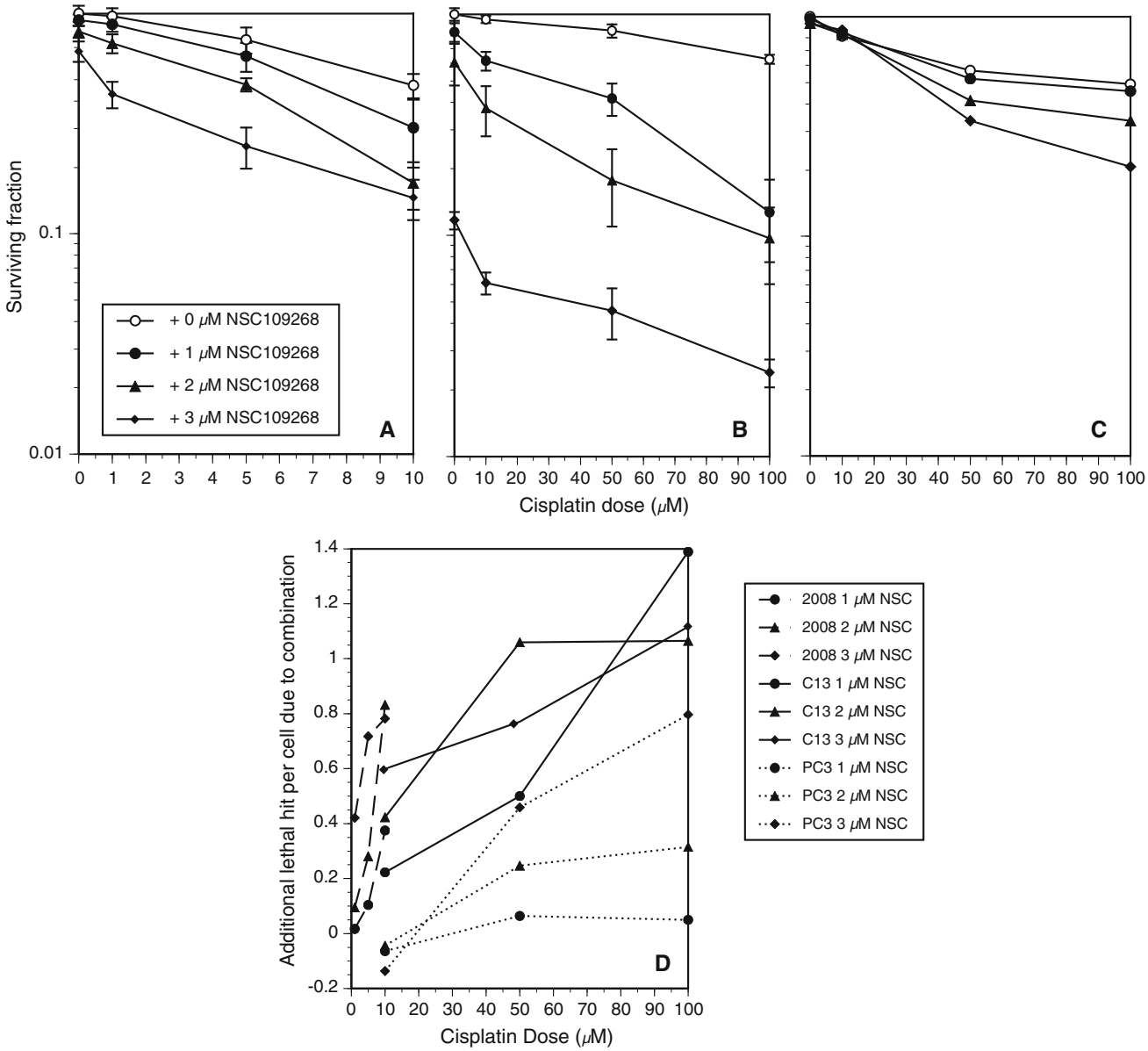

Fig. 4 MTS assays for cancer cell lines treated with CP with or without NSC109268. **a** 2008, **b** 2008/C13 ovarian cancer lines and **c** PC3 line were treated with CP and various concentrations of NSC109268 in serum-free medium, as indicated. OD_{490} readings were converted to cell numbers according to calibration curves and expressed as a fraction of cell number of untreated samples. *Vertical bars* in **a**, **b** depict standard errors. A representative single experiment is shown in **c**. **d** Survival values were converted into average lethal hits per cell, and additional hits arising from drug combination were plotted as a function of treatment

NSC109268 delays S-phase traversal of 2008/C13 ovarian cancer cells

Next, we wished to determine if any effects on cell cycle progression in human cancer cells resemble those found in yeast. FACS analysis performed at 48 h of culture revealed accumulation of CP-treated 2008/C13 cells in G2/M (Fig. 5). NSC109268 alone led to a slight increase in S-phase cells. In combination with CP, a much higher percentage of cells with apparent S-phase content were evident. Analysis of samples taken at an earlier time point (24 h) revealed that these cells had not exited prematurely from G2/M arrest but instead had not reached G2/M (Fig. 5). Thus, as in yeast, NSC109268 delays progression through S-phase in CP-treated ovarian cancer cells.

Discussion

Cisplatin and its derivatives are highly effective anticancer drugs, especially for treatment of testicular and ovarian cancer; however, their value is considerably diminished by

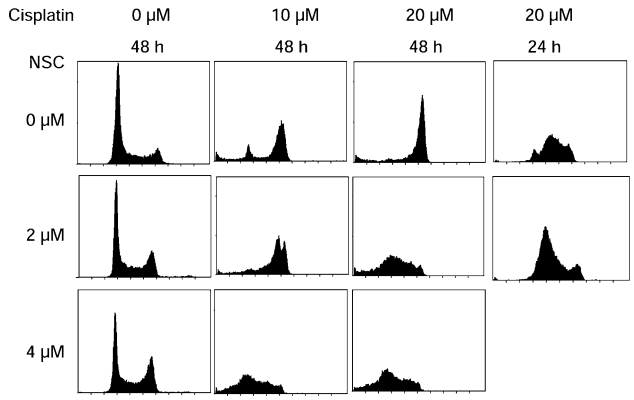

Fig. 5 Flow cytometric analysis of cell cycle distribution of 2008/C13 cells at 48 or 24 h of culture, treated with CP (10, 20 μM), NSC109268 (2, 4 μM) or a combination

the frequent development of resistance [28]. CP is thus a prime example of an anticancer drug whose efficacy might be greatly enhanced by an adjuvant, if initial resistance can be circumvented and acquired resistance can be overcome. Such an adjuvant could be the organic copper compound NSC109268, identified by us in yeast, and the closely related compound NSC109272. Initially, NSC109268 was isolated by us as a checkpoint-modifying drug of the topoisomerase inhibitor camptothecin for which it increases cellular resistance. The same was found for other DNA-damaging agents such as nitrogen mustard (shown in this study) or MNNG (unpublished) but, intriguingly, the opposite was true for platinum agents.

In both yeast and ovarian cancer cells, we consistently found that NSC109268 delays or inhibits progression of CP-treated cells through S-phase. This alteration of cell cycle kinetics and not the observed pronounced G2 arrest has previously been correlated with responsiveness to CP therapy [11].

For NSC109268, two activities have been described in the literature—inhibition of proteasomes [5, 6] and of phosphatase 2Cα [27]. Accumulation of polyubiquitinated substrates and inhibition of proteasomal chymotrypsin-like activities have been observed for NSC109268. The use of inhibitors of protein degradation is indeed an active area of anticancer research, and various possible targets have been discussed [30]. Enhancement of CP sensitivity by proteasome inhibitors has been described and correlated with enhanced adduct levels, diminished repair, deubiquitination of ubiquitinated histone H2A, increased p53 levels and accelerated apoptosis [21].

An appealing underlying mechanism for such sensitization is the prevention of degradation of Ctr1 [12], the main copper influx protein which is also largely responsible for CP uptake in yeast and mammalian cell [10]. Ctr1 is quickly downregulated in the presence of copper or CP by micropinocytosis and proteasomal degradation [8, 9, 17]. More efficient degradation may thus be a reasonable explanation for the enhanced resistance of 2008/C13 cells. The observed higher sensitivity of this cell line toward NSC109268 may somehow correlate with the underlying alterations of the proteasomal pathway.

Bound copper ions represent a remarkable feature of the identified compound [5]. However, competitive inhibition of CP efflux by copper ions is unlikely to be the underlying mechanism of sensitization. On the contrary, data from yeast indicate that CP treatment in the presence of copper ions results in *reduced* killing due to competition for uptake [10].

Besides drug uptake, several possible targets of proteasomal inhibitors have been associated with DNA damage repair and tolerance [22]. Interestingly, CP-treated 2008/C13 cells have been described as being more efficient in translesion DNA synthesis [20], which could also be a consequence of enhanced proteasomal degradation. In yeast, proteasomal mutants are altered in UV damage tolerance by lesion bypass [26]. Since DNA-damage-induced ubiquitination of PCNA and possibly other proteins are essential events associated with lesion bypass [7], absence of ubiquitin recycling may explain such an adverse effect of proteasome inhibitors. The slowed S-phase traversal following NSC109268 treatment of CP-exposed cells we consistently observed in yeast and 2008/C13 cells may reflect such inhibition of translesion synthesis. Interestingly, a cancer cell specificity of proteasome inhibitors in preventing translesion bypass of cisplatin damage has been demonstrated [29].

The second known activity of NSC109268, its inhibition of phosphatase 2Cα by binding to its active site, was discovered by in silico screening [27] and has been confirmed in vivo (T.J. Bartosh and R. Roque, unpublished). Type 2C phosphatases are required in yeast for checkpoint deactivation by dephosphorylating Rad53 [15], and type 2C phosphatase Wip1 may play a similar role in human cells by dephosphorylating p53, ATM/ATR and p38 [18]. Short-term, prolonged checkpoint arrest may ensue from inhibiting this process which may explain the observed S-phase slowdown. Its long-term inhibition may ultimately result in a lowered threshold for apoptosis or senescence. However, neither NSC109268 nor NSC109272 was identified in a screen of the diversity set for inhibitors of Wip1 [3].

Furthermore, yeast phosphatase 2A is also required for Rad53/Chk2 dephosphorylation [23], perhaps depending on the nature of the specific insult. The latter was found to play this role after CP treatment of ovarian cancer cells and to diminish resistance if downregulated [16, 24]. Still, phosphatases as a relevant target of NSC109268 should not be dismissed. While more specific for phosphatase 2Cα, some inhibition of phosphatase 2A by NSC109268 was observed in vitro [27].

In summary, the organic copper compounds NSC109268 and NSC109272 are promising agents for increasing CP sensitivity and overcoming CP resistance. Their known activity as proteasome or phosphatase inhibitors may explain the observed inhibition of S-phase progression that appears to be a vulnerable step in the cellular CP response. In contrast, the lethality of other types of agents may be reduced by slowing replication. Similarities of the effects in human cancer cells identify yeast as a valuable model to further analyze the molecular mechanism of these drug interactions that may be exploited for anticancer therapy.

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