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ORIGINAL CONTRIBUTIONS

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EFFECTS OF ALVOCIDIB AND CARBOPLATIN ON OVARIAN CANCER CELLS *IN VITRO*

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***Aim*: Failure of platinum chemotherapy is an unresolved issue in ovarian cancer. Targeted therapy has been added to the treatment options in solid cancers. Alvocidib is a cyclin dependent kinase inhibitor. *Materials and methods*: This study evaluated the effects of alvocidib together with carboplatin on ovarian cancer cells (BG-1 and Skov-3) *in vitro* applying proliferation assays, cell cycle distribution analyses, apoptosis induction assays, and drug accumulation assay. *Results*: Proliferation of both cell lines was inhibited by carboplatin and alvocidib. The interaction index revealed drug synergism at distinct drug concentrations. Cell cycle distribution was altered. Alvocidib induced apoptosis in Skov-3 cells, and necrosis in BG-1 cells. Rhodamine accumulation was increased by alvocidib or both compounds together. *Conclusion*: These data provide evidence for antiproliferative effects of alvocidib on human ovarian cancer cells *in vitro* associated with changes in cell cycle distribution, the induction of apoptosis, and modulation of intracellular drug accumulation. Alvocidib and carboplatin showed some cooperative activity.**

***Key Words*: flavopiridol, alvocidib, carboplatin, ovarian cancer, drug accumulation, apoptosis.**

Therapy of advanced ovarian cancer consists of cytoreductive surgery and systemic platinum containing chemotherapy [1]. However cancer recurrence and progression in the majority of patients remain pressing unresolved issues [2]. Clinically, chemoresistant and chemosensitive ovarian cancer can be distinguished differing in their responsiveness to platinum containing chemotherapy [3]. Targeted therapy has been added to the treatment options in ovarian cancer [4–5].

Carboplatin is the leading compound in systemic ovarian cancer therapy [6–7]. Clinical and experimental data have been accumulated to clarify the mechanisms of platinum treatment failure. Multidrug resistance proteins, ABC-transporters, a variety of cellular signalling pathways, decreased susceptibility to apoptosis induction, mutations in tumor suppressor genes and oncogenes have been demonstrated to mediate at least in part treatment failure and drug resistance [8–10].

Alvocidib, also known as flavopiridol, is a cyclin dependent kinase (cdk) inhibitor, specific for cdk-1, -2, -4 and -6; and is also a highly potent inhibitor of cdk-9 [11]. Alvocidib has been shown to inhibit cancer cell proliferation in a variety of experimental models [12, 13]. Alvocidib is active by competitively inhibiting ATP binding to multiple cdks including cdk-2, cdk-4, cdk-6 and cdk-7, whereas other molecular mechanisms are in effect in cdk-9 inhibition [14]. Inhibition of cdk-7 and cdk-9 strongly alters the cellular transcription machinery especially of those mRNA species with short half lives [15]. Alvocidib induced apoptosis in AO ovarian cancer cells *in vitro* and *in vivo* [16]. Alvocidib has been introduced into clinical trials for different solid tumors including recurrent ovarian cancer [17, 18].

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Abbreviations used: cdk – cyclin dependent kinase; IC – inhibitory concentration.

In this report, the effects of carboplatin and the cdk inhibitor alvocidib were investigated with respect to inhibition of cellular proliferation, modulation of cell cycle distribution, induction of apoptosis, and measurement of intracellular drug accumulation in human ovarian cancer cell lines *in vitro*.

MATERIALS AND METHODS

Drugs. Alvocidib was kindly provided by "Sanofi", Frankfurt, Germany. A stock solution of 10 mM was prepared in ethanol. Carboplatin was obtained from "Sigma", Steinheim, Germany. A stock solution of 10mM was prepared in DMSO.

Cell lines. Skov-3 and BG-1 human ovarian cancer cells were kindly provided by The German Cancer Research Center, Heidelberg, Germany. Cells were maintained in RPMI 1640 medium containing L-glutamine, 10% heat inactivated fetal calf serum ("Biokrom", Berlin, Germany; the other cell culture compounds were derived from "PAA Laboratories", Pasching, Austria) and penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37°C in a humidified atmosphere with 5% CO_2.

Proliferation assay. Cell proliferation assays and analysis were performed as described by F. Leonessa et al. [19]. Cells (10^4/ml) were distributed into 96 well plates ("Greiner", Frickenhausen, Germany) and allowed to adhere for 24 h. Medium was replaced by fresh medium containing vehicle or treatment reagents at the indicated concentrations. After the indicated treatment period, cells were fixed by adding 10% glutamatealdehyde ("Merck", Darmstadt, Germany) solution and stained by 0.05% crystal violet ("Sigma") in 25% methanol. Following washing three times with double distilled water, cell bound crystal violet was dissolved in 0.1 M sodium citrate and measured at 560 nm ("ELISA Reader, Tecan", Grödig, Austria). Experiments repeated in triplicate.

Calculation of drug synergism. Using the proliferation assay method described before, different

concentrations of carboplatin and alvocidib were combined. Cells were treated for 96 h, cell number was measured as before. The interaction index was calculated as described by R.J. Tallarida [20]. The interaction index (y) is calculated: $y = a/A + b/B$, where *a* and *b* represent the concentrations used in combination to achieve a distinct antiproliferative effect, and A and B represent the concentrations of either compound alone to achieve the same effect. $Y < 1$ indicates drug synergism. Experiments repeated in triplicate.

Cell cycle analysis. Skov-3 or BG-1 cells were distributed into 6-well plates (Greiner) and allowed to adhere for 24 h. Medium was replaced by fresh medium containing vehicle or treatment reagents at the indicated concentrations. Following an incubation for 96 h cells were harvested and fixed in 70% ethanol ("Roth", Karlsruhe, Germany) at 4 °C for one hour. Following washing and treatment with RNAse (1 mg/ml stock solution, "Serva", Heidleberg, Germany) at 37 ° for 20 min cells were stained with 0.01 mg/ml propidium iodine ("Calbiochem", Darmstadt, Germany) in PBS (PAA Laboratories) containing 0.01% NaN_3 and 2% FCS for 30 min at room temperature and light protected. Cell cycle distribution was determined using a FACS Calibur (BD Biosciences) and analysed using the Cell Quest Pro software (BD).

Apoptosis assay. Cells (1×10^5/well) were distributed into 6-well dishes and allowed to adhere for 24 h. Medium was replaced either containing vehicle or indicated treatment compounds. Cells were incubated for 48 h. Medium was removed, cells were harvested, free floating cells and collected adherent cells were washed and precipitated. Propidium iodine and annexin V staining and FACS (BD) detection were performed as described by the manufacturer (Roche, Mannheim, Germany).

Drug accumulation assay. Drug accumulation is a result of concurrent influx and efflux [21]. Rhodamine 123 is a substrate for ABC-transporter proteins [22]. Accumulation of rhodamine 123 can be modulated by substances which either compete as substrates for the transporter proteins or modulate the expression and biological activity of transporter proteins. Cells ($2{,}5 \times 10^5$/well) were distributed into 6-well dishes and allowed to adhere for 24 h. Medium was replaced containing either vehicle or indicated treatment compounds. Rhodamine 123 (0.25 μg/ml; "Sigma") was added, and rhodamine accumulation was measured after an incubation period of 60 min. Cells were harvested. Following propidium iodine staining, intracellular accumulated rhodamine 123 was measured via FACS (488 nm excitation, 530 nm emission, 570 nm filter). Benzylisothiocynate (100 nM) served as positive control for increasing rhodamine 123 accumulation [23].

RESULTS

Inhibition of proliferation by single compounds. Time dependent growth inhibition of Skov-3 cells *in vitro* by alvocidib (1 μM) was demonstrated. Incubation for up to eight days resulted in significant inhibition of proliferation (Fig. 1). The proliferation of BG-1 ovarian cancer cells was similarly decreased by alvocidib (1 μM) during the incubation period (Fig. 1). Inhibition of Skov-3 and BG-1 cell proliferation by alvocidib was concentration dependent (Fig. 1). For alvocidib, the IC50 was 0.01 μM and 0.015 μM in BG-1 and Skov-3 cells, respectively.

Carboplatin was shown to inhibit proliferation of both cell lines in a time and dose dependent manner (Fig. 2). For carboplatin, the IC 50 was 8 μM and 40 μM in BG-1 and Skov-3 cells, respectively.

Synergism of carboplatin and alvocidib. Fixed concentrations of one drug were combined with increasing concentrations of the second compound and vice versa to evaluate the interaction of carboplatin and alvocidip. Cells were incubated for 96 h, at least three independent experiments were performed for each combination.

Representative experiments are shown in Fig. 3. Fixed concentrations of alvocidib (ranging from 0.1 nM to 100 nM) were combined with increasing concentrations of carboplatin. Interaction indices for different combinations were calculated. For BG-1 cells, only moderate synergism was revealed, the lowest interaction index was 0.81. In Skov-3 cells, carboplatin (10 or 50 μM) combined with 10 nM alvocidib resulted in an interaction index of 0.4 and 0.52.

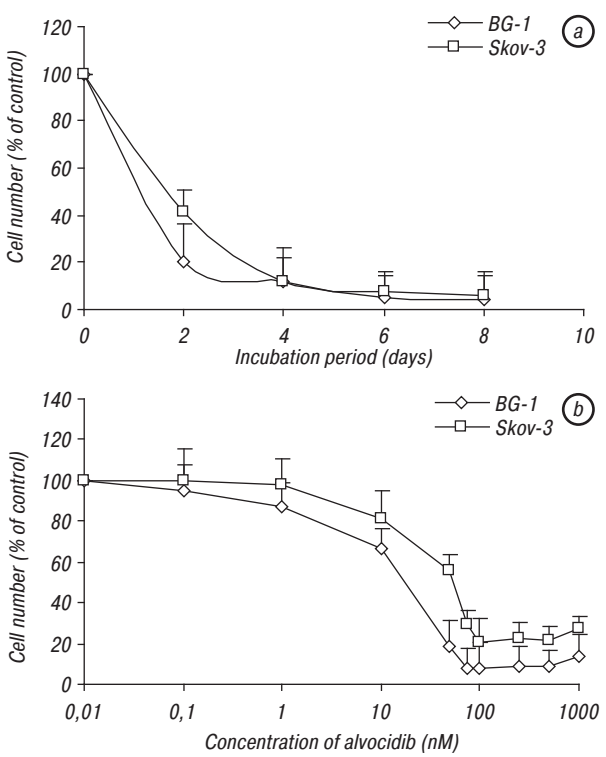

Fig. 1. Time (*a)* dependent inhibition of BG-1 and Skov-3 human ovarian cancer cell proliferation *in vitro* by 1 μM alvocidib incubated for the indicated time. Concentration (*b)* dependent inhibition of BG-1 and Skov-3 human ovarian cancer cell proliferation *in vitro* by alvocidib incubated for 96 h. Cells were incubated for indicated time in 96-well culture dishes, cell content was evaluated by crystal violet staining. Data represent mean and standard deviation of three independent experiments

Effects on cell cycle distribution. Treatment of Skov-3 cells with alvocidib (10 nM or 40 nM) for 96 h did not substantially alter cell cycle distribution (Fig. 4). Carboplatin (5 μM or 10 μM) increased proportion of cells in G2-phase, this effect was associated with a decrease of cells in G1. The combination of alvocidib and carboplatin resulted in a loss of cells in G1-phase.

Treatment of BG-1 cells with alvocidib (40 nM) for 96 h resulted in a decrease of cells in G1 and G2-phase of the cell cycle (Fig. 4). Carboplatin (5 μM or 10 μM) induced a minor decrease of cells in G1-phase. The combination of alvocidib with carboplatin resulted in a larger decrease of cells in G1 phase than treatment with carboplatin alone.

Induction of apoptosis. Annexin V staining was measured as a parameter for the induction of apoptosis. Apoptosis was neither induced by carboplatin (10 μM), nor by alvocidib as single agent (20 nM or 40 nM), nor by combination of both agents in BG-1 cells (Fig. 5). Necrosis was the leading cause of cell death in BG-1 cells.

Skov-3 cells were susceptible to the induction of apoptosis by carboplatin (5 μM or 10 μM) or alvocidib (20 nM or 40 nM) as single agents. Carboplatin together with alvocidib increased the induction of apoptosis compared to single agent treatment (Fig. 5).

Rhodamine 123 accumulation. Intracellular rhodamine 123 accumulation was determined as a model for cytotoxic drug accumulation. In BG-1 cells, carboplatin (10 μM) and alvocidib (0.1 or 1.0 μM) incubation resulted in increased rhodamine accumulation (Fig. 6). The effects were quantified relatively to the benzylisothiocynate (100 nM) induced rhodamine accumulation. Carboplatin (10 μM) together with 0.1 μM alvocidib increased rhodamine accumulation. 1 μM alvocidib combined with 10 μM carboplatin resulted in a higher rhodamine accumulation than carboplatin alone. The accumulation, however, was lower than induced by 1 μM alvocidib treatment.

In Skov-3 cells, carboplatin (10 μM) and alvocidib (0.1 or 1.0 μM) incubation resulted in increased rhodamine accumulation (Fig. 6). Combining carboplatin (10 μM) with 0.1 μM or 1 μM alvocidib revealed a further increase of rhodamine accumulation (Fig. 6).

DISCUSSION

In this report further evidence is added demonstrating the antiproliferative potential of alvocidib in ovarian cancer *in vitro*, and supporting the feasibility to combine alvocidib with carboplatin. For the first time, the data indicate cooperative and even modest synergistic effects of alvocidib and carboplatin *in vitro*.

Skov-3 and BG-1 cells are sensitive to the antiproliferative activity of carboplatin. Nevertheless, development of decreasing platinum sensitivity and eventually drug resistance is a phenomenon observable *in vitro* mimicking the clinical situation [24]. Skov-3 and BG-1 cell clones resistant to platinum

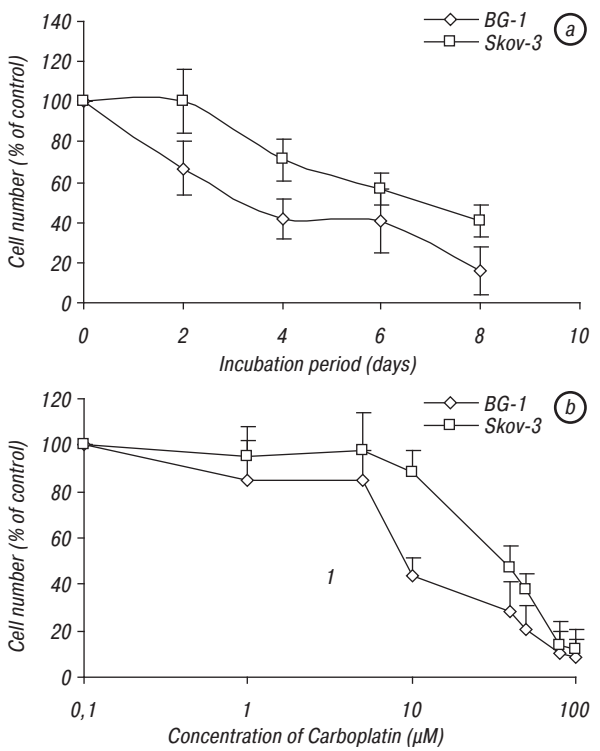

Fig. 2. Time (*a)* dependent inhibition of BG-1 and Skov-3 human ovarian cancer cell proliferation *in vitro* by 10 μM carboplatin incubated for the indicated time. Concentration (*b)* dependent inhibition of BG-1 and Skov-3 human ovarian cancer cell proliferation *in vitro* by carboplatin incubated for 96 h. Cells were incubated for indicated time in 96-well culture dishes, cell content was evaluated by crystal violet staining. Data represent mean and standard deviation of three independent experiments

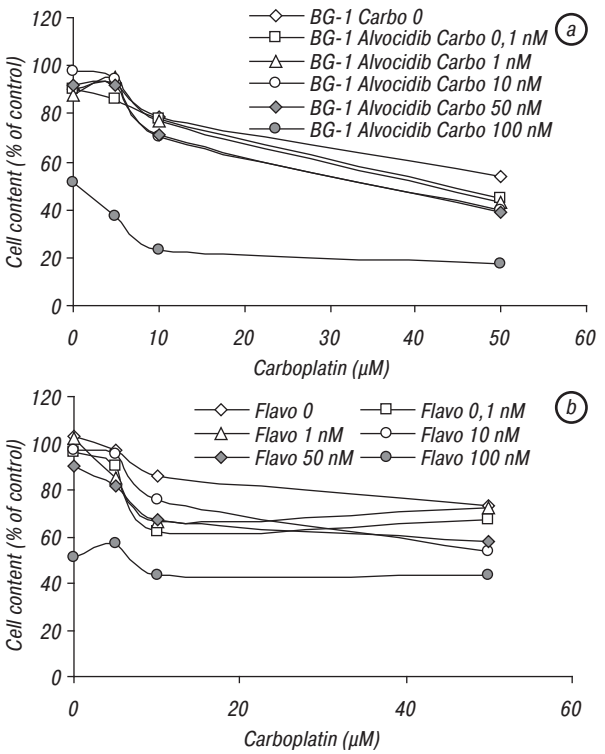

Fig. 3. Combined effects of carboplatin and alvocidib on BG-1 cells (*a*). Increasing concentrations of carboplatin were combined with distinct concentrations of alvocidib. Combined effects of carboplatin and alvocidib on Skov-3 cells (*b*). Increasing concentrations of carboplatin were combined with distinct concentrations of alvocidib. Cells were incubated for 96 h in 96-well culture dishes, cell content was evaluated by crystal violet staining. Data show the mean of three independent experiments. Standard deviation was <15%

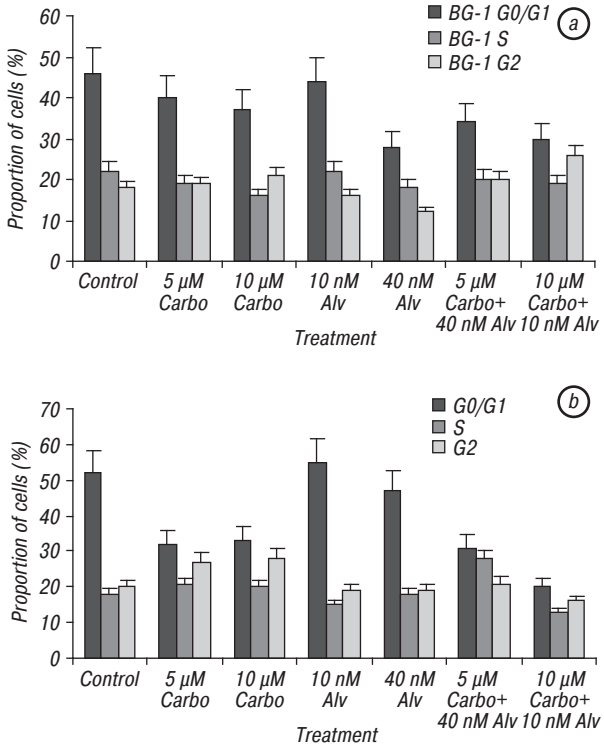

Fig. 4. Effects of carboplatin (Carbo) and alvocidib (Alv) on BG-1 cell cycle distribution (*a*). Effects of carboplatin and alvocidib on Skov-3 cell cycle distribution (*b*). Cells were incubated for 96 h with indicated compound(s) in 6-well culture dishes. 1 = control; 2 = 5 μM carboplatin; 3 = 10 μM carboplatin; 4 = 10nM alvocidib; 5 = 40 nM alvocidib; 6 = 5 μM alvocidib + 40 nM alvocidib; 7 = 10 μM carboplatin + 10 nM alvocidib. At the end, adherent and floating cells were harvested and stained with propidium iodine. FACS analysis was performed. Data show mean and standard deviation of three independent experiments

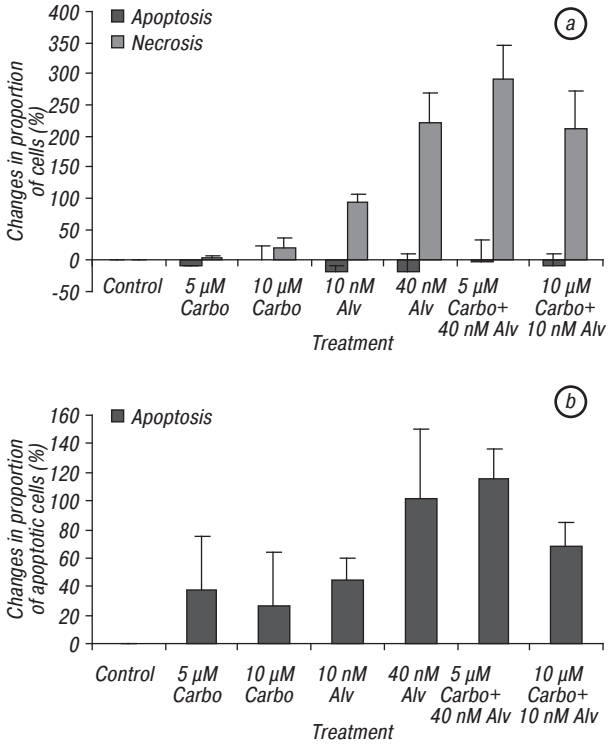

Fig. 5. Effects of carboplatin (Carbo) and alvocidib (Alv) on apoptosis and induction of necrosis in BG-1 cells (*a*). Effects of carboplatin and alvocidib on induction of apoptosis in Skov-3 cells (*b*). Cells were incubated for 48 hours with indicated compound(s) in 6-well culture dishes. 1 = control; 2 = 5 μM carboplatin; 3 = 10 μM carboplatin; 4 = 10nM alvocidib; 5 = 40 nM alvocidib; 6 = 5 μM carboplatin + 40 nM alvocidib; 7 = 10 μM carboplatin + 10 nM alvocidib. At the end, adherent and floating cells were harvested and annexin V staining was performed according to protocol. Propidium iodine counterstaining allowed the identification of necrotic cells. FACS analysis was performed. Data show mean and standard deviation of two independent experiments.

treatment have been established [25]. Alvocidib inhibited the proliferation of BG-1 and Skov-3 cells in a time and dose dependent manner. The IC50 values were below 50 nM of alvocidib with respect to inhibition of anchorage dependent growth. The cells were less sensitive than OV202 cells [26]. In a different *in vitro* model, alvocidib at a concentration of 100 nM or lower inhibited cell proliferation without induction of apoptosis, and sensitised the cells to apoptotic stimuli like tumor necrosis factor [27]. The alvocidib concentrations required to induce growth inhibition of BG-1 and Skov-3 ovarian cancer cells are in the range reported for different cell lines. It is noteworthy, that resistance to alvocidib is known in ovarian cancer cell lines [26].

It is shown for the first time, that distinct concentrations of alvocidib and carboplatin exerted some synergistic antiproliferative effects on BG-1 and Skov-3 cells. The positive cooperation of alvocidib with chemotherapy was also shown in breast or gastric cancer cells [28, 29].

Alvocidib is a cyclin dependent kinase inhibitor, that is active by competitively inhibiting ATP binding to multiple cdks including cdk-2, cdk-4, cdk-6 and cdk-7, whereas other molecular mechanisms are in effect in cdk-9 inhibition [14]. Inhibition of cdk-7 and cdk-9 strongly alters the cellular transcription machinery especially of those mRNA species with short half lives [14]. Effects induced by alvocidib include the induction of apoptosis, of cell differentiation, and antiangiogenesis [14, 30, 31]. The modulation of the Akt-signalling pathway is an important molecular pathway in the induction of apoptosis [13].

Alvocidib together with carboplatin induced necrosis in BG-1 cells. Skov-3 cells were susceptible to apoptosis-induction by alvocidib alone, and by the combination of alvocidib and carboplatin. Carboplatin induced apoptosis in Skov-3 cells as single agent. Cell cycle analysis and annexin V staining detected both, apoptosis and cytotoxic effects, induced by carboplatin and alvocidib. Earlier observations for different platinum derivatives showed a S-G2 block in BG-1 and CAOV3 ovarian cancer cells [32].

Additionally, the potential of alvocidib to influence drug resistance mechanisms was analysed. The experiments focused on drug accumulation in ovarian cancer cells. In OV202 cells reduced accumulation of cisplatin and flavopiridol was among the mechanisms mediating drug resistance [26]. The role of decreased drug accumulation as a mechanism of treatment resistance was further confirmed in different ovarian cancer cell lines, although the regulation of drug accumulation via membrane transporter proteins like MRP-1, MRP-3 and P-glycoprotein represent not the sole mechanism of therapy resistance [33].

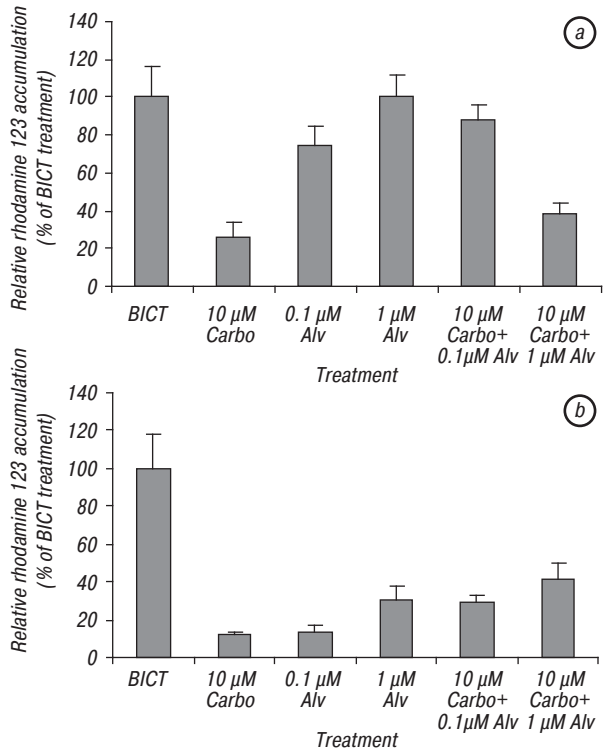

Fig. 6. Effects of carboplatin (Carbo) and alvocidib (Alv) on rhodamine 123 accumulation in BG-1 cells (*a*). Effects of carboplatin and alvocidib on rhodamine 123 accumulation in Skov-3 cells (*b*). BICT = Benzylisothiocynate (100 nM). 1 = 10 μM carboplatin; 2 = 0.1 μM alvocidib; 3 = 1 μM alvocidib; 4 = 0.1 μM alvocidib + 10 μM carboplatin; 5 = 1 μM alvocidib + 10 μM carboplatin. Cells were incubated for 60 minutes in the presence of rhodamine 123 and indicated treatment compound(s). Intracellular rhodamine accumulation was measured by FACS. Data show mean and standard deviation of two independent experiments

A role of MDR-1 and MRP proteins in human ovarian cancer with respect to prognosis and treatment response were described [8, 34–36]. Measuring intracellular rhodamine 123 accumulation is an established experimental model to evaluate the functional role of MDR-1 and MRP-1 [37]. Carboplatin is substrate of MRP, paclitaxel is a substrate of p-glycoprotein [38, 39]. In both cell lines, alvocidib increased rhodamine accumulation, BG-1 cells were more sensitive to this effect. This effect is shown for the first time in both cell lines tested. In addition to substrate competition, carboplatin was shown to inhibit MDR-1 gene expression [38]. These observations are of high interest with respect to paclitaxel or anthracycline containing chemotherapy in ovarian cancer. A recently published phase II trial strongly support the investigations of alvocidib with chemotherapy in ovarian and primary peritoneal cancer [40].

In summary, alvocidib as single agent significantly inhibited proliferation of BG-1 and Skov-3 ovarian cancer cells *in vitro*, induced changes in cell cycle distribution, necrosis and apoptosis, and increased rhodamine 123 accumulation. In part, positive cooperative effects of alvocidib together with carboplatin were detected. Our data together with published results by others indicate alvocidib as an exciting compound for basic and clinical research in ovarian cancer.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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