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In vitro investigations of new therapeutic agents on bladder tumor cell lines

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Abstract In this study sensitivity of human transitional cancer cells to the anticancer agent paclitaxel, an antimicrotubular drug, and to gallium nitrate, a group IIIa metal, was compared to that of the standard MVAC (methotrexate, vinblastine, doxorubicin and cisplatin) drugs. The reduction of cell proliferation was evaluated after 48 h of incubation of six different cell lines with each agent using the mean transit time (MTT) assay. We investigated both monolayers and spheroids. Paclitaxel showed significantly higher growth inhibitory effects on monolayers than vinblastine, both agents targeting the antimicrotubular apparatus. This could not be reproduced on spheroids, where a survival fraction of 50% was observed even at high concentrations (10 μM). High concentrations of gallium nitrate were needed to achieve sufficient toxicity. These concentrations are beyond the concentration achievable by systemic application. Our findings suggest that paclitaxel may be a clinically useful agent for systemic and intravesical use in bladder cancer.

Key words Paclitaxel · Gallium nitrate · MTT test · Chemotherapeutics · Bladder tumor cell line

Transitional cell carcinoma (TCC) is a chemosensitive malignant tumor with objective responses to a number of conventional chemotherapeutic drugs. The combination of methotrexate, vinblastine, doxorubicin and cisplatin (MVAC) is standard treatment in many institutions, with response rates of 39–57% [3, 10, 23]. However, the median survival of patients with advanced disease is about 12.5 months, and a long-term disease-free survival is achieved in only 4–15% of patients [12, 13, 18]. Tumor progression is common after chemotherapy, pointing to the importance of evaluating new anticancer therapeutics on this tumor. Studies have identified several new drugs with single-agent activity in urothelial carcinoma, and some phase II studies have already been conducted in patients with advanced disease [5, 24, 26]. However, there are few in vitro data available with regard to the cytotoxicity of these new drugs in TCC cell lines [17]. We studied the effects of paclitaxel and gallium nitrate in comparison to the standard drugs methotrexate, vinblastine, doxorubicin and cisplatin in six immortal, low-grade TCC cell lines derived from bladder cancer. Paclitaxel is a naturally occurring taxane, isolated from the bark of the Western yew, *Taxus brevifolia*. Its mechanism of action involves stabilization of microtubules and promotion of their assembly, unlike the action of other chemotherapeutics that target the microtubular apparatus, such as the vinca alkaloids. Paclitaxel has demonstrated a broad range of preclinical activity [1, 7] and appears to have manageable toxicities in phase I clinical trials [25]. Phase II trials of paclitaxel have demonstrated significant activity in ovarian cancer [15], breast cancer [9], and non-small-cell lung cancer [4]. Phase II trials of paclitaxel in metastatic transitional carcinoma are now in progress. Gallium nitrate is a group IIIa metal, and the antiproliferative effects are related to its binding to transferrin and subsequent ability to interfere with cellular iron metabolism as well as its inhibition of ribonucleotide reductase activity [14, 21]. Initial responses were reported by the Southwest Oncology Group [5]. A second phase I-II trial of metastatic transitional carcinoma showed a 17.4% response rate [20]. In combination with 5-fluorouracil, a 50% response rate was observed.

The aim of the present study was to determine the in vitro activity of paclitaxel and gallium nitrate in comparison to the standard MVAC drugs in a panel of human bladder-tumor cell lines and to identify characteristics of these agents that might prove useful for intravesical or systemic therapy of bladder cancer.

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Material and methods

Cell lines

All six human bladder tumor cell lines were derived from cystectomy specimens with a high tumor burden, pathologically classified as low-grade transitional carcinoma. After mechanical disaggregation, cells were placed in 100-mm plastic Petri dishes incubated at 37°C in a 100% humidified atmosphere containing 6% CO_2. Cell cultures were fed biweekly with fresh RPMI 1640 medium containing 13% fetal calf serum. Passage was performed when an 80–90% confluence was reached. In this study cells > eighth passage were used. Cells were characterized by positive immune histochemical staining for cytokeratin 20 and by an aneuploid DNA pattern in flow cytometry. Subcultures of all tumor cells were stored in a liquid nitrogen tank in 90% fetal calf serum and 10% dimethylsulfoxide for future study. Before the investigations started trypsination of a confluated Petri dish was performed, the cell suspension was counted and the viability was determined by trypan blue exclusion.

Spheroids

Spheroids are more representative of the three-dimensional in vitro situation than monolayers. These factors are cell-to-cell adhesion, extracellular matrix, catabolic products and reduced oxygen consumption as a result of high O_2 consumption, leading to a decrease in pH gradient and an increase in anaerobic glycolysis. Using spheroids, a more complex tumor model can be stimulated which lies between monolayers and a solid tumor.

Five thousand cells were placed in 96-well plates, each well being base coated with 0.5–1% agarose. DMEM growth medium was used. About 600 µm of the maximal diameter could be achieved. After being placed in the well each cell line was incubated for 48 h with the standard drugs methotrexate, vinblastine, doxorubicin and cisplatin in increasing concentrations. In addition, the same tests were performed with paclitaxel and gallium nitrate.

Each cell line was incubated with each drug for 48 h at concentrations from 0 µM to 10 µM. Drug incubation was started directly after placing the cells in the microwells. All drug testing was done at six dose levels using six plates/dose and the results were compared with those obtained for control plates. All drug assays were repeated at least 3 times to ensure reproducibility. Survival fraction (SF, percentage of inhibitory effect compared to control wells) was determined by the MTT test. This test is based on the capacity of viable cells to reduce transparent MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] to the blue-colored formazan, an index of mitochondrial enzyme activity. The quantity of staining was determined photometrically ($\lambda = 490$ nm). A reduction of the blue-stained cells is thus proportional to a reduced survival fraction. In addition, trypan blue exclusion and microcolorimetric investigations as described previously [2, 6, 11] were performed to verify the results obtained by the MTT assay. These control tests were done at the IC_{50} (concentration that achieved 50% growth inhibitory effect evaluated by the MTT assay). A quantity of 150 000 cells was exposed to the defined IC_{50} concentrations and heat output (microwatts) was measured.

The spheroids growing in the microwells were incubated with the increasing doses evaluated in the monolayer experiments and again the survival fraction was measured using the MTT test. The expected blood serum levels in vivo using the recommended doses of all drugs were obtained from the literature. Survival fraction (SF) at concentrations achievable by systemic administration was compared. Statistical analysis to compare the different agents at defined concentrations was done using SAS (Statistical Analysis System, SAS, Carry, USA) *Wilcoxon's test.*

Results

The concentration at a 48-h drug exposure was evaluated using paclitaxel, gallium nitrate, methotrexate, vinblastine, doxorubicin and cisplatine. Tumor cells were exposed to these drugs at concentrations ranging from 0.01 to 10 µM. Vinblastine was compared to paclitaxel because it is an antimicrotubular agent that induces microtubule disassembly and it has known activity in bladder cancer. Figure 1 depicts the mean results of all experiments with monolayers. With increasing doses of the individual agents, a reduction of SF to less than 10% was achieved. The mean IC_{50} for paclitaxel, vinblastine, gallium nitrate, methotrexate, cisplatine and doxorubicin was 0.003, 0.006, 2, 0.006, 0.1, and 0.001 µM, respectively.

Paclitaxel is significantly more active at a concentration of 0.01 µM than vinblastine, the mean SF for paclitaxel and vinblastine being 57% and 77% respectively. The growth inhibitory effects of vinblastine and paclitaxel on monolayers and spheroids are compared in Fig. 2. Although a reduction of cell proliferation was achieved in the spheroid model, this was significantly lower than in the monolayer system. High concentrations (> 1 µM) which achieve an IC_{90} in the monolayers reduce the SF to about 60% in the spheroid model. Both

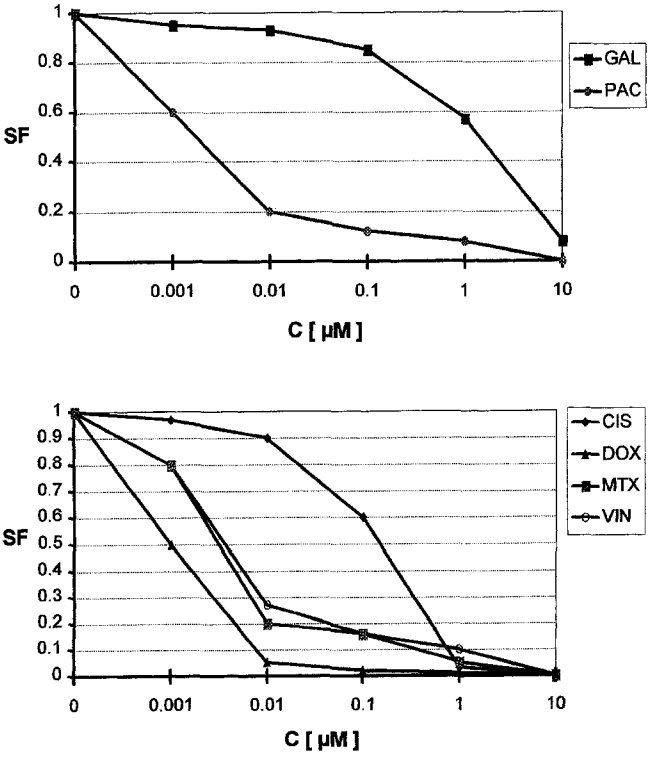

Fig. 1 SF of all agents on monolayers. *Lower diagram* depicts the standard MVAC drugs. *CIS* cisplatin, *DOX* doxorubicin, *MTX* methotrexate, *VIN* vinblastine. *Upper diagram* shows paclitaxel (*PAC*) and gallium nitrate (*GAL*). Whereas all agents achieve sufficient reduction of SF at 1 µM, gallium nitrate has an SF of > 50%, which is statistically significant

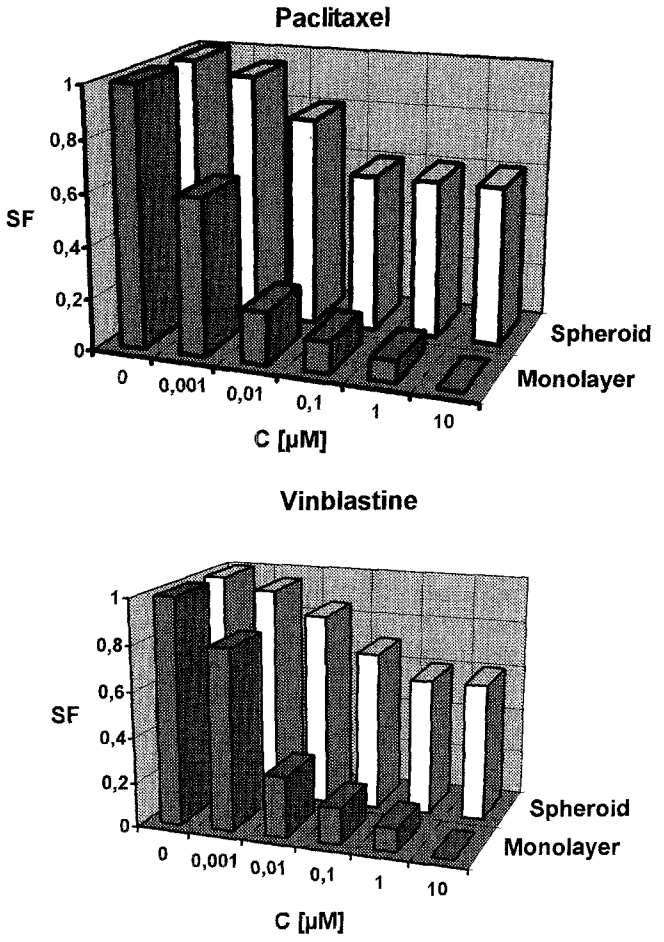

Fig. 2 SF of paclitaxel and vinblastine on monolayers and spheroids. Paclitaxel has a significant higher cell inhibitory effect up to 0.01 µM on monolayers. SF of both drugs on spheroids is significantly higher than on monolayers

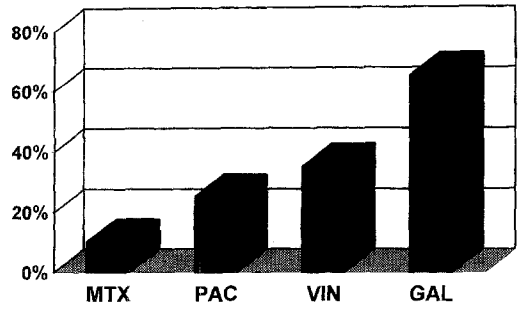

Fig. 3 SF at concentrations within the clinically achievable range for systemic application

vinblastine and paclitaxel showed the same effects. Comparing the SF of the monolayers at concentrations that are within the achievable range of systemic administrations in vivo, the mean SF for vinblastine was 35% and for paclitaxel 25% (Fig. 3). After increasing the concentrations to $10 \times IC_{90}$, the spheroids disintegrated and cell death was observed. The mean IC_{50} of gallium nitrate is 2 µM. At a concentration of 1.2 µM, the expected blood serum level in vivo, a SF of 65% was observed in the monolayer tumor model.

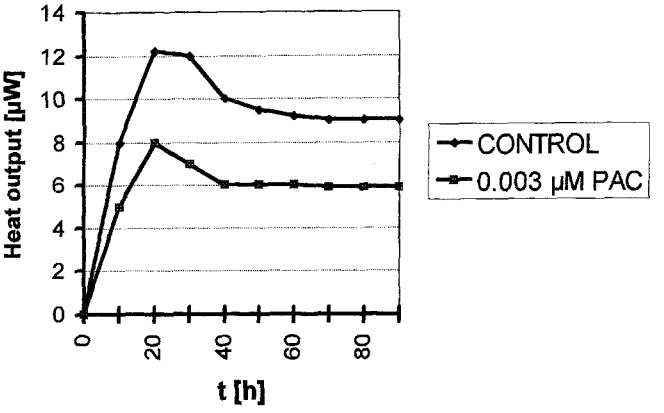

Fig. 4 Mean microcalorimetric results obtained from six cell lines comparing 48-h incubation of 1.5×10^5 cells with 0.003 µM paclitaxel (*PAC*) with a control sample. Heat output is approximately half in 0.003 µM paclitaxel

Control testing of the MTT assay using trypan blue exclusion confirmed the results. At the IC_{50}, cell density was verified to be half that of the control wells without cytotoxic agent. Microcalorimetric evaluation revealed approximately half the heat output in cell samples incubated with the IC_{50}. Figure 4 shows the mean microcalorimetric results from control cells compared to cells incubated with 0.003 µM paclitaxel (IC_{50}).

Discussion

Paclitaxel was highly active against all the panels of tumor cells used in this study. The activity occurred within the clinically achievable range for systemic administration of this drug. These findings suggest paclitaxel may have significant clinical activity in bladder cancer. The eastern cooperative oncology group (ECOG) conducted a phase II trial in patients with advanced transitional carcinoma who had not received prior therapy. Of the 26 patients, 11 (42%) had an objective response, with 7 patients (27%) achieving a complete response [18, 19]. Rangel et al. [17] have shown paclitaxel to be more active than vinblastine in nine bladder tumor cell lines. Our investigations demonstrated that paclitaxel is active at lower concentrations than vinblastine.

The antiproliferative effect of gallium nitrate has been known since the early 1970s [8]. The first published clinical trial by the Southwest Oncology Group in 1991 [8] showed an objective initial response of 31%. There are no data available of tests of the antiproliferative effects of gallium nitrate on bladder tumor cell lines. In our experiments gallium nitrate was found to have minimal activity in vitro at similar clinically achievable drug levels. At a concentration of 1.2 µM, which is the expected blood serum level in vivo, a SF of 65% was observed.

The penetration of drugs has been studied by Nederman and Twentyman [16]. Drugs require about 2 h to penetrate efficiently through the spheroid. An incubation time of 48 h is far beyond this time. In our investigations a significantly higher SF was observed in all

agents in the spheroid tumor model compared to the monolayers. High concentrations (> 1 μM) that reduced the SF to less than 10% in monolayers resulted in an SF of more than 60% of the cells in spheroids. Previous investigations [16] have shown drugs diffuse within minutes through a spheroid. The high percentage of unaffected cells cannot be explained by reduced concentrations in the center of the spheroid. We exposed all the spheroids to concentrations evaluated to be highly cytotoxic for monolayers but found that the spheroids were much more resistant. After increasing the concentrations to $10 \times IC_{90}$ evaluated in monolayers, the spheroids disintegrated and cell death was observed. This suggests that cell-to-cell adhesion may increase resistance to cytotoxic agents. The three-dimensional growth of cells in spheroids, giving rise to oxygen and proliferation gradients and other tumor-like properties, has been suggested to be responsible for spheroid resistance to drug effects [22]. Distribution of cells in the proliferation cycle, gradients of oxygen, glucose, and other nutrients, and accumulation of toxic products of metabolism are some examples of the better simulation of a solid tumor compared to monolayers.

The response rate to MVAC is 39% in clinical trials [12], median survival is 12.5 months, and only 4% of patients are long-term disease-free survivors. Our difference in survival fraction between monolayers and spheroids may explain the high initial response rate of transitional carcinoma cells and the high long-term failure rate. Here, despite the initial high response rates, the long-term failure rate is high in patients with advanced transitional carcinoma.

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